

Date: July 22, 1998

Case Docket No. MEGAN 100 CON

Express Mail No. EM 500 209 588 US

07/22/98
 1c540 U.S. PTO
 09/120970
 07/22/98

Patent Commissioner for Patents
 Washington, D.C. 20231

Transmitted herewith for filing is the patent application of

Inventors: Roy Curtiss, III and Steve A. Tinge

Serial No.: Continuation of 08/473,789

For: RECOMBINANT BACTERIAL VACCINE SYSTEM WITH ENVIRONMENTALLY LIMITED VIABILITY

Enclosed are:

- ☒ 1 Page of Abstract, 56 Pages of Specification, 5 Pages of Claims, and 6 sheets of informal drawings.
- ☒ An Assignment of the invention to Washington University
- ☒ Copies of Verified Statement (Declaration) Claiming Small Entity Status by Megan Animal Health, Inc. and Washington University.
- ☒ An unexecuted Declaration and executed Declaration.
- ☒ A Preliminary Amendment.
- ☒ Request for Filing a Continuation Application under 37 CFR §1.53(b).

The filing fee has been calculated as shown below:

| | (Col. 1) | (Col. 2) |
|---|-----------|-----------|
| FOR: | NO. FILED | NO. EXTRA |
| BASIC FEE | | |
| TOTAL CLAIMS | 35-20 = | 15 |
| INDEP CLAIMS | 3-3 = | 0 |
| <input type="checkbox"/> MULTIPLE DEPENDENT CLAIM PRESENTED | | |

| SMALL ENTITY | |
|--------------|-------|
| RATE | FEE |
| | \$395 |
| x 11 = | \$165 |
| x 41 = | \$0 |
| x135 = | \$0 |
| TOTAL | \$560 |

| OTHER THAN A SMALL ENTITY | |
|---------------------------|-------|
| RATE | FEE |
| | \$790 |
| x 22 = | \$ |
| x 82 = | \$ |
| x270 = | \$ |
| TOTAL | \$ |

* If the difference in Col. 1 is less than zero, enter "0" in Col. 2.

- ☐ Please charge my Deposit Account No. 01-2507 in the amount of \$. A duplicate copy of this sheet is enclosed.
- ☒ A check in the amount of \$560.00 is enclosed.
- ☒ The Commissioner is hereby authorized to charge payment of the following fees associated with this communication or credit any overpayment to Deposit Account No. 01-2507. A duplicate copy of this sheet is enclosed.
 - ☒ Any additional filing fees required under 37 CFR 1.16.
 - ☒ Any patent application processing fees under 37 CFR 1.17.
- ☒ The Commissioner is hereby authorized to charge payment of the following fees during the pendency of this application or credit any overpayment to Deposit Account No. 01-2507. A duplicate copy of this sheet is enclosed.
 - ☒ Any patent application processing fees under 37 CFR 1.17.
 - ☐ The issue fee set in 37 CFR 1.18 at or before mailing of the Notice of Allowance, pursuant to 37 CFR 1.311(b).
 - ☒ Any filing fees under 37 CFR 1.16 for the presentation of extra claims.

Respectfully submitted,

[Signature]
 Robert A. Hodges, Reg. No. 41,074

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Roy Curtiss, III and Steve A. Tinge

Serial No: Continuation of 08/473,789

Express Mail Label No.
EM 500 209 588 US

Filed: July 22, 1998

Date of Deposit: July 22, 1998

For: *RECOMBINANT BACTERIAL VACCINE SYSTEM WITH
ENVIRONMENTALLY LIMITED VIABILITY*

Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Prior to examination, please amend the application as follows. This Preliminary Amendment is being filed along with a Continuation Application filed under 37 C.F.R. § 1.53(b). It is believed that no fee is required with this submission. However, should a fee be required, the Commissioner is hereby authorized to charge any additional fees to Deposit Account No. 01-2507.

Amendment

In the Specification

On page 1, after the title "RECOMBINANT BACTERIAL VACCINE SYSTEM WITH ENVIRONMENTALLY LIMITED VIABILITY" and before line 4, which begins "The United States Government...", please insert as a new paragraph

Continuation of U.S.S.N. 08/473,789

Filed: July 22, 1998

PRELIMINARY AMENDMENT

-- This application is a continuation of copending Application Serial No. 08/473,789, filed June 7, 1995, entitled "RECOMBINANT BACTERIAL VACCINE SYSTEM WITH ENVIRONMENTALLY LIMITED VIABILITY," by Roy Curtiss, III and Steve A. Tinge. Application Serial No. 08/473,789, filed June 7, 1995, is hereby incorporated herein by reference.--

Respectfully submitted,



Robert A. Hodges
Reg. No. 41,074

Date: July 22, 1998

ARNALL GOLDEN & GREGORY LLP

2800 One Atlantic Center

1201 West Peachtree Street

Atlanta, Georgia 30309-3450

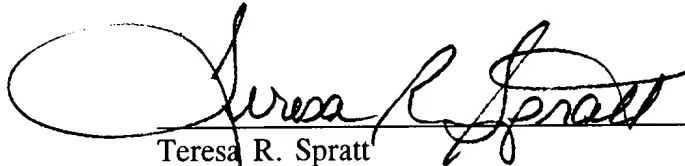
(404) 873-8796

(404) 873-8797 (fax)

Continuation of U.S.S.N. 08/473,789
Filed: July 22, 1998
PRELIMINARY AMENDMENT

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.10

I hereby certify that this Preliminary Amendment and any documents referred to as attached therein are being deposited with the United States Postal Service on this date, July 22, 1998, in an envelope as "Express Mail Post Office to Addressee" service under 37 C.F.R § 1.10, Mailing Label Number EM 500 209 588 US addressed to Box Patent Application, Assistant Commissioner of Patents and Trademarks, Washington, D.C. 20231.


Teresa R. Spratt

Date: July 22, 1998

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Roy Curtiss, III and Steve A. Tinge

Serial No: Continuation of 08/473,789

Express Mail Label No.
EM 500 209 588 US

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For: *RECOMBINANT BACTERIAL VACCINE SYSTEM WITH
ENVIRONMENTALLY LIMITED VIABILITY*

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

**REQUEST FOR FILING A
CONTINUATION APPLICATION UNDER 37 C.F.R. § 1.53(b)**

Sir:

This is a request for the filing of a continuation application under 37 C.F.R. § 1.53(b) of pending prior application Serial No. 08/473,789, filed June 7, 1995, by Roy Curtiss, III and Steve A. Tinge for "Recombinant Bacterial Vaccine System with Environmentally Limited Viability".

I hereby verify that the attached papers are a true copy of what is shown in my records to be the above-identified prior application. The application includes 1 page of Abstract, 56 pages of Specification, 5 pages of claims, and 6 sheets of informal drawings.

A copy of the assignment to Washington University as filed in the prior application and recorded at Reel 7600, Frame 0008 on May 6, 1993.

Continuation of U.S.S.N. 08/473,789
Applicants: Roy Curtiss, III and Steve A. Tinge
Filed: July 22, 1998
REQUEST FOR FILING A CONTINUATION
APPLICATION UNDER 37 C.F.R. § 1.53(b)

The power of attorney in the prior application is to Patrea L. Pabst. Madeline I. Johnston, and Robert A. Millman. Also enclosed is an Associate Power of Attorney Under 37 C.F.R. § 1.34.

The inventorship for the claims in the continuation application is the same as in the prior application.

Verified Statements that this application as filed by a small entity have been filed in the parent application and such status is still proper and desired.

A check in the amount of \$560.00 is enclosed to cover the filing fee for the continuation application for a small entity. It is believed that this is the proper filing fee since the continuation application will include 3 independent claims and a total of 35 claims after entry of the preliminary amendment.

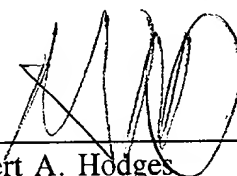
Please preliminarily amend the application in accordance with the attached Preliminary Amendment. The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 01-2507. To facilitate this process, applicants have enclosed a duplicate copy of this Request.

This application is being filed on July 22, 1998, by mailing the application to Box Patent Application, Commissioner for Patents and Trademarks, Washington, D.C. 20231 via the United States Postal Service "Express Mail Post Office to Addressee" Service under 37 C.F.R. § 1.10. The Express Mail Label No. EM 500 209 588 US appears in the heading of this paper, which is attached to the application, pursuant to 37 C.F.R. § 1.10(b).

Continuation of U.S.S.N. 08/473,789
Applicants: Roy Curtiss, III and Steve A. Tinge
Filed: July 22, 1998
REQUEST FOR FILING A CONTINUATION
APPLICATION UNDER 37 C.F.R. § 1.53(b)

I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,



Robert A. Hodges
Reg. No. 41,074

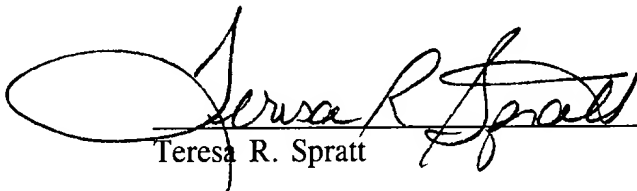
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REQUEST FOR FILING A CONTINUATION
APPLICATION UNDER 37 C.F.R. § 1.53(b)

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.10

I hereby certify that this Request For Filing a Continuation Application Under 37 C.F.R. §1.53(b) and any documents referred to as attached therein are being deposited with the United States Postal Service on this date, July 22, 1998, in an envelope as "Express Mail Post Office to Addressee" service under 37 C.F.R § 1.10, Mailing Label Number EM 500 209 588 US addressed to Box Patent Application, Assistant Commissioner of Patents and Trademarks, Washington, D.C. 20231.


Teresa R. Spratt

Date: July 22, 1998

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Roy Curtiss, III and Steve A. Tinge

Serial No: Continuation of 08/473,789

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Date of Deposit: July 22, 1998

For: *RECOMBINANT BACTERIAL VACCINE SYSTEM WITH
ENVIRONMENTALLY LIMITED VIABILITY*

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

**REQUEST FOR FILING A
CONTINUATION APPLICATION UNDER 37 C.F.R. § 1.53(b)**

Sir:

This is a request for the filing of a continuation application under 37 C.F.R. § 1.53(b) of pending prior application Serial No. 08/473,789, filed June 7, 1995, by Roy Curtiss, III and Steve A. Tinge for "Recombinant Bacterial Vaccine System with Environmentally Limited Viability".

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08/473,789-01

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The inventorship for the claims in the continuation application is the same as in the prior application.

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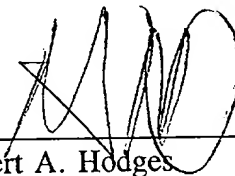
Please preliminarily amend the application in accordance with the attached Preliminary Amendment. The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 01-2507. To facilitate this process, applicants have enclosed a duplicate copy of this Request.

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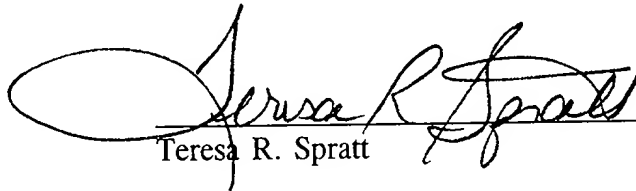
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Teresa R. Spratt

Date: July 22, 1998

**APPLICATION
FOR
UNITED STATES LETTERS PATENT**

**BY
ROY CURTISS III
AND
STEVEN A. TINGE**

**FOR
RECOMBINANT BACTERIAL VACCINE SYSTEM
WITH ENVIRONMENTALLY LIMITED VIABILITY**

RECOMBINANT BACTERIAL VACCINE SYSTEM WITH ENVIRONMENTALLY LIMITED VIABILITY

The United States Government has rights to this invention
5 pursuant to PHS Grants DE06669, AI24533, and HD29099.

Background of the Invention

The invention relates to recombinant microorganisms with
environmentally limited growth and viability, and more particularly to
10 recombinant microorganisms that will not grow outside of a host organism.

Genetically engineered microorganisms have widespread utility
and importance. For example, they can be used to produce foreign proteins,
and thus can be used industrially for synthesis of products such as
interferons, insulin, and growth hormone; they can also be used as vaccines
15 to produce an immune response. However, it is undesirable for the
genetically-engineered microorganism to persist in the environment.

Physical containment was the first means used to control the
spread of genetically engineered microorganisms. More sophisticated means
were then developed where microorganisms were contained by introducing
20 debilitating mutations that prevent their growth in the absence of specific
growth conditions, such as a particular amino acid. U.S. Patent No.
4,190,495 discloses microorganisms with multiple mutations to prevent
growth or genetic transfer outside of the controlled laboratory conditions.

Suicide plasmids have been described for use in biological
25 containment of microorganisms (Molin *et al.*, *Annual Review of Microbiology*
47:139-166 (1993)). Suicide plasmids generally use differential expression of
a specific gene in and out of a controlled environment to prevent survival of
the microorganism outside of the controlled environment. The use of suicide
plasmids or systems has been limited to the expression of a required gene, or
30 the repression of a lethal gene, under laboratory or other artificial conditions.

In the absence of the supplied stimulus, the lethal gene becomes expressed. Either event leads to cell death either directly or due to a severe competitive disadvantage. Most suicide systems depend upon expression of a gene that is actively toxic to the microorganism. A common problem with active suicide systems is the high selective pressure for mutations in the killing gene. This is analyzed, for example, by Knudsen and Karlström, *Applied and Environmental Microbiology* 57(1):85-92 (1991), who used a suicide function controlled by LacI^q binding to *Plac* to repress expression of *relF*, thus preventing the suicide that would be caused by synthesis of the *relF* gene product. Expression of RelF, and thus display of the suicide phenotype, required the addition of IPTG. This system therefore served as a laboratory model to study induction of host killing within the plasmid-containing host or after transfer of the plasmid to a host lacking the *lacI* encoded repressor. The efficacy of the system was limited on the one hand by mutations that made killing by the *relF* gene product ineffective, and on the other hand by low level expression of *relF* even in the absence of added IPTG such that cells in the culture grew slowly.

Molin *et al.*, *Bio/Technology* 5:1315-1318 (1987), describes the use of *hok*, encoding a small peptide that is lethal when expressed in many bacterial species, to prevent survival of a recombinant microorganism when outside of a controlled fermenter environment. The *hok* gene is homologous to the *relF* gene. Molin *et al.* (1987) uses an invertible promoter to control expression of *hok*. Stochastic inversion of the inactive promoter to the active orientation causes the bacterial population to dwindle due to the death of a predetermined fraction of the cells per unit time. This stochastic cell death is dependent on the relative expression of the *fimB* and *fimE* genes which control the flip-flop orientation of the invertible promoter. This system does not lead to a rapid drop in bacterial population density except under ideal laboratory conditions.

Molin and Kjelleberg, *AMBIO* 22(4):242-245 (1993), and Ramos *et al.*, *Bio/Technology* 13:35-37 (1995) have hypothesized that microorganisms for release in natural environments might be regulated by suicide genes. Molin and Kjelleberg describe the use of a suicide gene regulated by general or specific starvation. Ramos *et al.* disclose the use of *gef* family genes, of which *hok* and *relF* are members, and nuclease genes, as suicide genes. Ramos *et al.* also described killing by these gene products expressed upon IPTG induction of the inducible *Plac* fused to the *gef* gene. Ramos *et al.* describe regulation of the suicide function by loss of a specific nutrient or condition, such as occurs outside of artificially controlled conditions, and by linking the regulatory stimulus to the task of the microorganism. This was accomplished by controlling the rate of inversion of a promoter which in one orientation causes expression of *relF*, resulting in cell death. This system, of course, only leads to a gradual loss in the viability of the cell population once the cells are in an environment lacking a nutrient. Ramos *et al.* also suggests the use of biological containment to make live vaccines feasible. However, Ramos *et al.* (page 37) points out that the complexity of the human gut precludes the design of control circuits based on specific stimuli, suggesting containment based on differential growth rates in and out of the gut. Ramos *et al.* does not suggest any regulatory system that could achieve this goal.

Live bacterial vaccines have been described that express a desired antigen and colonize the intestinal tract of animals (Curtiss *et al.*, *Curr. Topics Micro. Immun.* 146:35-49 (1989); Curtiss, *Attenuated Salmonella Strains as Live Vectors for the Expression of Foreign Antigens*, in *New Generation Vaccines* (Woodrow and Levine, eds., Marcel Dekker, New York, 1990) pages 161-188; Schödel, *Infection* 20(1):1-8 (1992); Cárdenas and Clements, *Clinical Micro. Rev.* 5(3):328-342 (1992)). Most work to date has used avirulent *Salmonella typhimurium* strains synthesizing various

foreign antigens for immunization of mice, chickens and pigs. Several avirulent *S. typhi* vectors have been evaluated in human volunteers (Tacket *et al.*, *Infect. Immun.* 60:536-541 (1992)) and several phase I clinical trials with recombinant avirulent *S. typhi* strains are in progress in the U.S. and Europe.

5 Although research progress toward expanding and further improving the recombinant avirulent *Salmonella* antigen delivery strategy has progressed at a reasonable rate, commercial development of recombinant vaccines for the control of infectious diseases of animals or humans has been slow. An important safety advantage of the live attenuated bacterial vaccine
10 vectors as compared to the use of viral vector based vaccines is the ability to treat an immunized patient with oral ciprofloxacin or amoxicillin, should an adverse reaction occur. However, current live bacterial vaccines have the disadvantage that oral administration leads to fecal shedding, with the potential risk that the bacterial vaccine strain will proliferate in nature and
15 infect individuals not selected for immunization. It is known, for example, that fecal coliforms can persist for extended periods under field conditions, with only moderate reductions in numbers (Temple *et al.*, *Appl. Environ. Microbiol.* 40:794-797 (1980)). There is also concern that these surviving vaccine strains will transmit their cloned genetic information to more robust
20 microorganisms encountered in nature with not always predictable consequences. Although the transmission of most expressed genes to wild-type microbial species would not be harmful, some recombinant vectors expressing genes for sperm-specific antigens or lymphokines could have adverse consequences if widely disseminated. It is therefore desirable to
25 have a biological containment system regulated by the conditions that differ between the target environment and other environments. In the case of live recombinant vaccines, it is desirable to have a microorganism that survives inside the animal, but dies outside of the animal. Live attenuated bacterial antigen delivery vectors with inherent biological containment features to

preclude survival, proliferation and gene transfer in nature would increase the acceptability and enthusiasm for use of this type of vaccine delivery system.

It is therefore an object of the present invention to provide an Environmentally Limited Viability System for use in controlling viability of
 5 targeted microorganisms and limiting the survival of recombinant extrachromosomal genetic information if transferred to other microorganisms.

It is another object of the invention to provide a live recombinant vaccine with environmentally limited viability.

It is a further object of the invention to provide a method of
 10 vaccination using a live recombinant vaccine with environmentally limited viability.

Summary of the Invention

Disclosed is an Environmentally Limited Viability System (ELVS) for
 15 microorganisms based on differences between permissive and non-permissive environments. Viability of the microorganisms are limited to the permissive environment by specifically expressing one or more essential genes only in the permissive environment, and/or expression of one or more lethal genes only in the non-permissive environment. Environmentally Limited Viability
 20 Systems are also disclosed involving coordinate expression of a combination of required genes and lethal genes. Microorganisms containing an Environmentally Limited Viability System are useful for release into a permissive environment. Temperature regulated Environmentally Limited Viability Systems are particularly suited for use with recombinant avirulent
 25 *Salmonella* vaccines for warm-blooded animals by limiting the vaccine growth to the warmer environment inside the animal. Such vaccines can be administered to immunize humans or warm-blooded animals against bacterial, viral, mycotic and parasitic pathogens, especially those that colonize on or invade through mucosal surfaces. This antigen delivery system can also be

A preferred example of a temperature regulated

This Environmentally Limited Viability System can be reinforced by introducing a temperature-regulated chromosomal *polA* gene, which is expressed at temperatures greater than about 30°C, especially at about 37°C, but not at temperatures below about 30°C, especially at about 25°C. Since

This Environmentally Limited Viability System can be reinforced by introducing a temperature-regulated chromosomal *polA* gene, which is expressed at temperatures greater than about 30°C, especially at about 37°C, but not at temperatures below about 30°C, especially at about 25°C. Since

the expression vector containing the *asd* gene requires expression of the *polA* gene for replication, the expression vector containing the *asd* gene will not be able to replicate at temperatures below about 30°C, thus leading to loss of the vector and cell death.

5

Brief Description of the Drawings

Figure 1 is a diagram of an Environmentally Limited Viability System including a plasmid and chromosomal components, using a temperature-regulated *asd* gene on a PolA-dependent plasmid. The arrows illustrate the interaction of the repressors and promoter/operator regions for lambda and P22 at low and high temperatures, while the +/- indicates the production of a functional gene product. The promoters and regulators depicted may be interchanged with other environmentally regulated systems as they are characterized.

15 Figure 2 is a diagram of *pir*-dependent suicide vector pMEG-011 which is unable to replicate in any host lacking a functional *pir* gene. The vector contains the multiple cloning site from the commercially available cloning vector pNEB193, providing a number of unique cloning sites and the blue/white screen associated with the *lacZ* α gene in an appropriate host.

20 The vector also contains the tetracycline resistance gene (*tetA*) from transposon Tn10, allowing selection for tetracycline resistance with integration into the chromosome and then selection against the integrated vector on media containing fusaric acid, facilitating replacement of wild-type genes with modified genes containing deletions and insertions.

25 Figure 3 is a diagram of the *pir*-dependent suicide vector pMEG-096 containing the bacteriophage P22 *c2* repressor gene (C2) operatively linked to the bacteriophage lambda promoter right (P_R) and the bacteriophage lambda temperature sensitive repressor *cl857* gene, both genes in a cartridge flanked by portions of the *S. typhimurium asd* gene (hatched

with diagonal lines). This arrangement allows allele replacement of the wild-type chromosomal *asd* gene of any strain with homology to the flanking *asd* gene segments.

Figure 4 is a diagram of a vector based Environmentally Limited Viability System (ELVS) with *S. typhimurium* host MGN-392 containing expression vector pMEG-104, the combination designated as strain MGN-417. pMEG-104 contains the *asd* gene of *S. typhimurium* operatively linked to the promoter left of bacteriophage lambda (λP_L), and the lysis genes of bacteriophage P22 (*lysI3* and *lysI9*) operatively linked to the promoter right of bacteriophage P22 ($P_{22.R}$). The promoter left of bacteriophage lambda and promoter right of bacteriophage P22 are placed in opposite orientations on the vector such that transcription of the promoter left of bacteriophage lambda will produce an antisense RNA of the lysis genes and transcription of the promoter right of bacteriophage P22 will produce an antisense RNA of the *asd* gene. Both genes are in a cartridge flanked by two different transcription terminators (*trpA* term and 5S T1 T2). pMEG-104 also contains the low copy-number, DNA polymerase I-dependent p15A origin of replication (*ori*). The host cell MGN-392 contains the *ci857* and *c2* repressor gene cartridge derived from pMEG-096 on its chromosome. This cartridge replaces a central portion of the chromosomal *asd* gene of the host cell. This cartridge provides thermally regulated limited viability when the plasmid pMEG-104 is present in this host cell. Bold arrows depict temperature-regulated expression of the vector components of the ELVS.

Figure 5 is a diagram of vector pMEG-100. This vector is the same as vector pMEG-104 except that pMEG-100 contains a kanamycin resistance gene.

Figure 6 is a graph of the growth (CFU/ml) versus time (hours) of *S. typhimurium* hosts with and without an Environmentally Limited Viability System. Filled squares indicate growth points of cells containing

pMEG-104 grown at room temperature. Open squares indicate growth points of cells containing pMEG-104 grown at 37°C. Filled triangles indicate growth points of cells containing the Asd⁺ vector pYA292 grown at room temperature. Open triangles indicate growth points of cells containing pYA292 grown at 37°C.

Detailed Description of the Invention

There are two main components required for a successful biological containment system. The first component consists of genes encoding products that are either essential or potentially fatal to the bacterial cells containing them. The second component is a regulatory system to turn the critical genes on or off at the appropriate time. The challenge presented by a containment system for a live bacterial vaccine is the recognition of an environmental trigger that will signal the death of the cell upon release into a non-permissive environment, but will not prevent growth in a permissive environment.

The disclosed Environmentally Limited Viability System combines specific regulation with essential and/or lethal genes to limit the viability of a microorganism to a permissive environment. The Environmentally Limited Viability System can be combined with other mutations to limit the virulence of the bacterial host. A containment system encoding both essential and lethal gene products requires the cell maintain the desired regulation or suffer either loss of an essential component or the effect of a lethal product. In the preferred embodiment, the essential gene and the lethal gene are extrachromosomal, for example, on a plasmid. However, any or all of the components of the Environmentally Limited Viability System can be located on a chromosome of the host microorganism.

The Environmentally Limited Viability System makes use of genetically engineered host cells which can be maintained as a genetically stable population, wherein the host cells express a desired expression

product. The host cells used for the Environmentally Limited Viability System contain environmentally regulated essential genes, lethal genes and/or replication genes. The expression of these genes is regulated to allow expression of the essential and replication genes only in a permissive environment, and expression of the lethal genes in non-permissive environments. Host cells used in the Environmentally Limited Viability System characteristically have an inactivated native gene encoding a gene essential for cell survival. A copy of this gene, which acts as an essential gene in the system, is placed on the vector of the system to provide selective pressure for maintenance of the vector.

The Environmentally Limited Viability System makes use of host cells adapted for use of the genetic components of the system. Vectors which are suitable for transforming the host cells, which contain the essential and lethal genes, and into which the gene encoding the desired polypeptide may be inserted, are also described. The disclosed Environmentally Limited Viability System is suitable for use for the production of desired polypeptides in industrial settings, for example, by growth in fermenters. For example, recombinant production of dangerous toxins can be performed with a reduced risk of escape by using an Environmentally Limited Viability System. Microorganisms incorporating the system may also be used as live vaccines.

Genetic regulatory systems suitable for use in the Environmentally Limited Viability System are those that modulate gene expression based on environmental conditions, such as temperature, osmolarity, pH, and oxygen availability. A basic motif of many of these regulatory systems is an environmentally based change in a trans regulatory element that alters its interaction with a control sequence of the regulated gene. Such systems can be adapted to regulate the genes of the Environmentally Limited Viability System based on environmental changes that define a permissive and non-permissive environment.

As used herein, permissive environment refers to an environment in which microorganisms incorporating an Environmentally Limited Viability System are viable. Such an environment might possess a key characteristic, such as a specific temperature, osmolarity, pH, or oxygen concentration. As used herein, a non-permissive environment refers to an environment in which microorganisms incorporating an Environmentally Limited Viability System are non-viable. As used herein, a non-viable cell or microorganism refers to a cell or microorganism that cannot grow. Viability is always considered relative to specific environments and environmental conditions. Thus, a cell is considered non-viable in a particular environment even though the cell would be viable in other environments.

A. Essential Genes

The Environmentally Limited Viability System makes use of essential genes to limit cell viability and to provide selective pressure for maintenance of the vector component of the system. An essential gene, as used herein, refers to a gene required for cell viability. Containment is provided by regulating the essential gene, such that the gene is expressed in a permissive environment but is not expressed in a non-permissive environment.

As used herein, "gene" refers to a nucleic acid sequence having a coding sequence operatively linked to a control sequence. "Coding sequence" refers to a nucleic acid sequence encoding RNA or protein. The RNA or protein encoded by a coding sequence is referred to as an expression product. A coding sequence can encode one or more expression products. "Control sequence" refers to DNA sequences which are necessary to effect the expression of coding sequences to which they are operatively linked. Generally such control sequences include a promoter and ribosome binding site. The term "control sequence" is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous, for example,

operators. "Operatively linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence operatively linked to a coding sequence refers to a control sequence ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

The essential gene or genes can be located on an extrachromosomal vector or on a chromosome of the microorganism. Placing regulated essential genes on the chromosome provides stability for the genes and allows multiple essential genes to be combined in a single Environmentally Limited Viability System without increasing the size and complexity of the vector portion of the system. It is preferred, however, that at least one essential gene be expressed on the vector portion of the system in order to provide selection for plasmid maintenance.

The essential gene is also used to maintain the vector component of the Environmentally Limited Viability System in microorganisms of the system. This is important because most plasmids are maintained through the use of antibiotic selection and in many natural environments the use of antibiotics is unworkable or undesirable. Maintenance of plasmids in recombinant hosts when present in natural environments preferably use a different selection system. A preferred selection method involves a balanced-lethal host-vector system, where an essential gene is carried on a vector and the chromosomal gene is deleted, creating a balanced-lethal condition. The "lethal" deletion is balanced by the presence of the vector borne copy of the wild-type gene.

Genes essential for metabolism or growth. Most genes encoding enzymes involved in the metabolism or growth of a microorganism can be used as essential genes. All that is required is that the gene is regulated so that it is expressed only in a permissive environment. It is preferred that the essential gene be derived from a gene in the host

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microorganism. In many bacteria, expression of modification methylases are required to prevent endogenous restriction endonucleases from cleaving the host chromosome. Thus a modification methylase gene can also be used as the essential gene. Genes required for nucleic acid replication, such as genes encoding DNA ligase and gyrase, are essential for cell growth and can be used as the essential gene.

Genes essential for cell wall or cell membrane integrity. All bacteria have a peptidoglycan layer of the cell wall which imparts shape and rigidity. The peptidoglycan is made of a polymer of repeating muramic acid-N-acetylglucosamine and is cross-linked by short peptides. In all gram-negative bacteria and in *Mycobacterium* and in *Nocardia* species of Eubacteria, the peptide is composed of L-alanine, D-glutamic acid, meso-diaminopimelic acid (DAP), and D-alanine. In most gram-positive microorganisms the DAP is replaced by its decarboxylation product L-lysine.

Enzymes which catalyze the biosynthesis of the cell wall component and its precursors are known in the art. For example, in the synthesis of peptidoglycans, the enzyme may be one which catalyzes the insertion of the cross-linking peptide, for example, D-alanyl-D-alanine ligase, or of the synthesis of the carbohydrate polymer, or it may be an enzyme which catalyzes a step in the biosynthesis of a precursor, for example, diaminopimelic acid (DAP). For a review of the biosynthesis of this family of amino acids, see Umbarger (1978). Examples of genes encoding enzymes which catalyze steps in the biosynthesis of DAP are known in the art for a variety of organisms, see, for example, *Genetic Maps* (O'Brien, ed., Cold Spring Harbor Laboratory, 1987), and include, for example, in *S. typhimurium* the *dapA* and *dapB* genes and for example, *E. coli*, the *dapA*, *dapB*, *dapC*, *dapD*, and *dapE* genes. Another enzyme which is essential for DAP synthesis is β -aspartate semialdehyde dehydrogenase (Asd), which is encoded by the *asd* gene.

The use of *asd* in an unregulated balanced-lethal system is described by (Nakayama *et al.*, *Bio/Technology* 6:693-697 (1988)). This system uses plasmid vectors with the wild-type gene encoding the enzyme β -aspartate semialdehyde dehydrogenase required for the synthesis of DAP in conjunction with a *Salmonella* strain which has a chromosomal *asd* mutation. Since DAP is synthesized only by bacteria and is not present in the tissues of an immunized host, loss of the Asd^+ vector from the recombinant avirulent *Salmonella* leads to death and lysis of the bacterial cell (Nakayama *et al.* (1988)). In the disclosed Environmentally Limited Viability System, the *asd* balanced-lethal system can be adapted by regulating the expression of the essential *asd* gene.

Accordingly, a preferred essential gene is *asd*, encoding β -aspartate semialdehyde dehydrogenase, an enzyme required for the synthesis of an essential component of the rigid layer of the bacterial cell wall, namely diaminopimelic acid (DAP). DAP is only synthesized by bacteria and is not prevalent in the environment. DAP is synthesized in six enzymatic steps from β -aspartate semialdehyde, which, in turn, is synthesized in two steps from L-aspartic acid. In the first step, L-aspartic acid is phosphorylated by one of several (usually three) β -aspartokinases which are encoded by several (usually three) separate genes regulated independently by repression and/or feedback inhibition of the gene products by the ultimate end products L-threonine, L-methionine, and L-lysine. β -aspartophosphate is converted in one step to β -aspartic semialdehyde by β -aspartic semialdehyde dehydrogenase, the product of the *asd* gene. Mutants with a point mutation in or deletion of the *asd* gene as well as mutants with mutations in any of the six genes specifying the enzymes for converting β -aspartate semialdehyde to DAP have an obligatory requirement for DAP in all media. When DAP-requiring mutants are deprived of DAP, they die and are lysed, releasing their contents.

The gene for β -aspartate semialdehyde dehydrogenase from *Streptococcus mutans* PS 14 (UAB62) has been cloned and expressed in *asd* mutants of *E. coli*, as described by Jagusztyn-Krynicka *et al.* (1982), and Curtiss *et al.* (1982). Subsequently, the *S. mutans asd* gene was sequenced
 5 by Cardineau and Curtiss (1987).

In both gram-positive and gram-negative bacteria, the peptide cross-linking repeating muramic acid-N-acetylglucosamine polymers contain D-alanine. D-alanine is synthesized from L-alanine by alanine racemase, the product of the *dal* gene (*B. subtilis*), and then is converted to a D-alanyl-D-alanine dipeptide by the enzyme D-alanyl-D-alanine ligase, the product of the
 10 *ddl* gene. D-alanine is coupled to the L-alanyl D-glutamyl DAP or L-alanyl-D-glutamyl-L lysine tripeptide which is attached to one muramic acid-N-acetylglucosamine polymer to form a pentapeptide. The terminal D-alanine is then cleaved during the enzymatic cross-linking reaction to the next
 15 muramic acid-N-acetylglucosamine polymer. Mutants of *Bacillus subtilis* lacking the ability to synthesize D-alanine or to synthesize D-alanyl-D-alanine lyse in media devoid of D-alanine or of the dipeptide. *dal* mutants of *B. subtilis* lacking alanine racemase have been isolated (Ferrari *et al.*, *Bio/Technology* 3:1003-1007 (1985); Dul *et al.* (1973)). *ddl* mutants lacking
 20 D-alanyl D-alanine ligase have been isolated in *E. coli* (Wijsman (1972), Miyakawa *et al.* (1972), Lugtenberg *et al.* (1973)) and in *B. subtilis*. As in the case of the *asd* and *dap* mutations, the inclusion of *dal* and/or *ddl* mutations in strains of bacteria limits the viability of the organisms, since such mutant strains are unable to survive in environments other than a
 25 carefully controlled laboratory environment.

All microorganisms have a cell membrane that retains the cytoplasmic contents. During growth, new membrane components must be synthesized to maintain the integrity of increased surface area of the membrane on the growing cell. Critical genes involved in metabolism of cell

membrane components can be used for the essential gene. Such genes include genes involved in fatty acid biosynthesis (*fab*), fatty acid degradation (*fad*), phospholipid synthesis (*pls*), and phospholipases.

B. Lethal Genes

5 Regulated lethal genes are used in the Environmentally Limited Viability System to actively kill host cells that escape to a non-permissive environment. A lethal gene, as used herein, refers to a gene that is lethal to the host microorganism when expressed in the host. A regulated lethal gene which is repressed in the permissive environment, but expressed in the non-
10 permissive environment, acts as a positive containment mechanism for the Environmentally Limited Viability System.

 Lethal genes useful in the Environmentally Limited Viability System include cell-killing gene products of the *gef* gene family that form holes in cell membranes when over-produced. Some of these host killing
15 genes, such as *hok*, are plasmid derived and ensure that plasmid-free cells do not survive (Gerdes *et al.*, *Proc. Natl. Acad. Sci. USA* 83:3116-3120 (1986)). Others, such as *gef* and *relF*, are of chromosomal origin with unclear functions (Poulsen *et al.*, *Mol. Microbiol.* 5:1639-1648 (1991), Gerdes *et al.*, *EMBO J.* 5:2023-2029 (1986), Gerdes *et al.*, *New Biol.* 2:946-
20 956 (1990)). Other bacterial host killing gene products useful as lethal genes include nucleases (Molin *et al.*, *Annu. Rev. Microbiol.* 47:139-166 (1983)), phospholipases (Givskov *et al.*, *J. Bacteriol.* 170:5855-5862 (1988)) and plasmid maintenance genes (Figurski *et al.* (1982)). Endolysins and holins of the bacteriophages lambda (Bienkowska-Szewczyk *et al.*, *Mol. Gen. Genet.*
25 184:111-114 (1981), Reader and Siminovitch, *Virology* 43:623-637 (1971)) and P22 (Rennell and Poteete, *Virology* 143:280-289 (1985)) are also available. Their expression forms lesions in the bacterial inner membrane with subsequent degradation of the cell wall and release of cytoplasmic contents (Young, *Microbiol. Rev.* 56:430-481 (1992)). Preferred lethal genes

are the combination of bacteriophage P22 lysis genes 13 and 19, coding for a porin and lysozyme, respectively. The joint action of these two proteins leads to production of holes in the cell membrane and degradation of the bacterial cell wall. In adapting the Environmentally Limited Viability System to various host cells, it is preferred that lysis genes from phage that normally infect the chosen host be used as the lethal genes.

A gene encoding a tRNA (including a suppressor tRNA), or a tRNA that would have acceptor activity but a wrong codon to cause synthesis of mutant proteins, could also be used as a lethal gene. A nuclease active against DNA or mRNA could also be lethal when overexpressed.

The lethal gene may be located on a chromosome of the host microorganism or an extrachromosomal element. It is preferred that lethal genes used in the Environmentally Limited Viability System be expressed on a vector. One advantage is to limit the amount of growth during which an inactivating mutation could occur.

It is preferred that the lethal gene be placed on an extrachromosomal vector regulated by a chromosomally expressed negative trans regulatory element. With this arrangement, should the plasmid be transferred to another microorganism, the lethal gene will be expressed constitutively and lead to the demise of that recipient cell. Thus, the Environmentally Limited Viability System will prevent both survival and spread of the recombinant strain within the environment and transmission of recombinant plasmids within natural populations of bacteria.

C. Replication Genes

Biological containment of the Environmentally Limited Viability System can be enhanced by regulating the expression of a replication gene. A replication gene, as used herein, refers to a gene required for replication of an extrachromosomal vector. Use of a properly regulated replication gene reinforces the dependence of the cell on an essential gene by placing the vector containing the essential gene into a host with a gene required for the

replication and maintenance of that vector. A preferred replication gene is *polA*, encoding DNA polymerase I, an enzyme required for replication of a number of plasmid replicons. The failure of the host cell to synthesize DNA polymerase I will halt plasmid vector replication and progeny cells will be formed lacking the plasmid containing the essential gene, thus resulting in non-viable cells (Kingsbury *et al.*, *J. Bacteriol.* 114:1116-1124 (1973)).

It is preferred that the environmentally regulated replication gene be placed on a chromosome of the host microorganism.

D. Regulatory Elements

The three categories of genes described above (essential, lethal, and replication) require a regulatory system that ensures viability in the permissive environment and rapid death and lysis in the non-permissive environment. Regulation is coordinated and maintained using control sequences either directly linked to the essential, lethal, and replication genes of the Environmentally Limited Viability System, or linked to coding sequences encoding trans regulatory elements that modulate the expression of the genes of the Environmentally Limited Viability System. The switch in expression is mediated by environmentally based changes in trans regulatory elements. In general, the genes of the Environmentally Limited Viability System can be regulated 1) by linking the coding sequences to control sequences that promote or prevent transcription in the permissive and non-permissive environments, 2) by regulating the expression of trans regulatory elements that in turn promote or prevent transcription of the genes of the Environmentally Limited Viability System, 3) by adapting or altering trans regulatory elements, which act on the genes of the Environmentally Limited Viability System, to be active or inactive in either the permissive or non-permissive environment, or using combinations of these schemes. The Environmentally Limited Viability System requires the use of various promoters to coordinate expression of different elements of the system.

Some elements, such as temperature-sensitive repressors or environment-specific regulatory elements, use constitutive promoters.

1. **Trans Regulatory Elements.** As used herein, trans regulatory element refers to a molecule or complex that modulates the expression of a gene. Examples include repressors that bind to operators in a control sequence, and antisense RNA that binds to and prevents translation of a mRNA. For use in Environmentally Limited Viability Systems, expression from regulated promoters is modulated by promoter regulatory proteins. These promoter regulatory proteins can function to induce or repress transcription from the promoter.

Another type of trans regulatory element is RNA polymerase. Genes of the Environmentally Limited Viability System can be regulated by linking them to promoters recognized only by specific RNA polymerases. By regulating the expression of the specific RNA polymerase, expression of the gene is also regulated. For example, T7 RNA polymerase requires a specific promoter sequence that is not recognized by bacterial RNA polymerases. A T7 RNA polymerase gene can be placed in the host cell, regulated to be expressed only in the permissive or non-permissive environment. Expression of the T7 RNA polymerase will in turn express any gene linked to a T7 RNA polymerase promoter. A description of how to use T7 RNA polymerase to regulate expression of a gene of interest, including descriptions of nucleic acid sequences useful for this regulation appears in Studier *et al.*, *Methods Enzymol.* 185:60-89 (1990).

Another type of trans regulatory element is antisense RNA. Antisense RNA is complementary to a nucleic acid sequence of a gene to be regulated. Hybridization between the antisense RNA and the target sequence prevent expression of the gene. Typically, antisense RNA complementary to the mRNA of a gene is used. Expression of the genes of the Environmentally Limited Viability System is regulated by controlling the expression of the antisense RNA. Expression of the antisense RNA in turn

prevents expression of the gene of interest. A complete description of how to use antisense RNA to regulate expression of a gene of interest appears in U.S. Patent No. 5,190,931.

Trans regulatory elements, such as repressors or antisense RNA, can be expressed from either the chromosome or a plasmid. To limit the size and complexity of the plasmid portion of the system, however, it is preferred that these regulatory elements be expressed from the bacterial chromosome.

2. Temperature Sensitive Regulation. A preferred type of regulation for microorganisms intended for growth in humans or warm-blooded animals is temperature regulation. This is based on the contrast between the high and constant body temperature present in mammals and birds and the low and variable temperature present in the ambient environment into which microorganisms are shed. To accomplish this, a preferred Environmentally Limited Viability System expresses genes ensuring survival at about 37°C and prevents expression of genes that would cause death or lysis at about 37°C. The system is designed so that at about 30°C or below, the genes needed for survival cease to be expressed and genes that actively cause cell death and lysis are expressed at high level. This can be accomplished by using promoters and regulatory elements that are regulated by temperature, or by adapting other regulatory systems to function in a temperature-dependent manner.

Temperature-regulated gene expression suitable for use in the Environmentally Limited Viability System are described by (Neidhardt *et al.*, *Annu. Rev. Genet.* 18:295-329 (1984)). There are well-defined heat shock genes (Neidhardt *et al.* (1984)) that are well expressed at high temperature. Although the expression of these genes is temperature-regulated, there is frequently some low basal level of expression at the restrictive temperatures (Jones *et al.*, *J. Bacteriol.* 169:2092-2095 (1987)). Temperature-regulated promoters exhibiting tighter control are described by (Tobe *et al.*, *Mol.*

Micro. 5:887-893 (1991), Hromockyi *et al.*, *Mol. Micro.* 6:2113-2124 (1991), Qoronfleh *et al.*, *J. Bacteriol.* 174:7902-7909 (1992)).

For essential genes, the *S. flexneri virB* promoter can be used, with *S. flexneri virF* gene and promoter elsewhere on the same plasmid, on a
 5 separate plasmid, or on the chromosome (Hromockyi *et al.* (1992); Tobe *et al.* (1991). A *Yersina* two component system for temperature regulation can also be used involving the structural gene for the temperature-regulated positive activator *virF* (Lambert de Rouvroit *et al.*, *Molec. Microbiol.* 6:395-409 (1992) in combination with promoters of the *yopH* or *yadR* genes, with
 10 or without modification of the histone-like YmoA protein encoded by *ymoA* (Cornelis, in *Molecular Biology of Bacterial Infections* (Cambridge University Press, Cambridge, 1992)). The *virF* gene is equivalent to *lcrF* in *Y. pestis* (Hoe *et al.*, *J. Bacteriol.* 174:4275-4286 (1992). Other promoters useful for expression of the essential gene can be identified by screening for
 15 cold-sensitive lack of expression of β -galactosidase in an *S. typhimurium lacZ* fusion library (Tanabe *et al.*, *J. Bacteriol.* 174:3867-3873 (1992)). Many other repressor-promoter combinations can be adapted to express essential genes in a temperature-specific manner by using temperature-sensitive forms of the repressor. Methods for obtaining temperature-sensitive mutant
 20 repressors are well established.

The containment ability of the temperature regulated system can be further augmented by using promoters that function optimally at low temperatures and have minimal activity at 37°C, so that these promoters can be engineered to regulate the expression of the lethal genes or a repressor of
 25 the essential genes at low temperatures. Utilization of these promoters provides alternate controls of lethal genes and an additional means to eliminate any residual activity due to low level expression of an essential or replication gene at low temperatures.

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Cold-specific expression can also be accomplished by coupling a gene to a cold-shock promoter or a cold-sensitive promoter. Cold shock promoters may be obtained from known cold-shock genes. Cold shock genes with promoters have been described (Jones *et al.* (1987)). Promoters with temperature-specific expression can be identified by a promoter probe vector. Such vectors have flanking DNA from a gene that is dispensable and which can readily be selected for or identified using, for example, a chromogenic substrate.

A preferred system that is less complex involves the interaction of the bacteriophage lambda promoters, λP_L and λP_R , with the *cI857* temperature-sensitive repressor. This system has been described, for example, by Lieb, *J. Mol. Biol.* 16:149-163 (1966). The lambda phage promoters λP_L and λP_R , with their mutant temperature-sensitive repressor *cI857*, provide a tightly regulated system used in expression vectors to provide controlled expression of toxic genes (O'Connor and Timmis, *J. Bacteriol.* 169:4457-4462 (1987)). The *cI857* gene product is synthesized but inactive at 37°C and is synthesized but actively represses expression of genes at 30°C and below whose transcription is controlled by either λP_L or λP_R .

Leaky expression from the control sequences of an Environmentally Limited Viability System, if encountered, can be eliminated in several ways. The level of cI repressor produced can be increased by placing the *cI857* gene under the control of a strong constitutive promoter, such as *P_{trc}*, thus providing an excess of the thermosensitive repressor. In addition, more binding sites for the cI repressor can be introduced within the operator region of λP_R to reduce transcriptional starts at non-permissive temperatures, or engineered into regions downstream of the promoter element to hinder transcription at lower temperatures. Additionally, an antisense RNA for the regulated gene could be transcribed from a differently regulated promoter oriented in the opposite direction to λP_R .

E. Vectors

The disclosed Environmentally Limited Viability System will employ, unless otherwise indicated, conventional techniques of cell culture, molecular biology, microbiology, and recombinant DNA, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (1989); *DNA Cloning*, Volumes I and II (Glover, ed., 1985); *Oligonucleotide Synthesis* (Gait ed., 1984); *Nucleic Acid Hybridization* (Hames and Higgins, eds., 1984); Perbal, *A Practical Guide To Molecular Cloning* (1984); the series, *Methods in Enzymology* (Academic Press, Inc.); Vectors: *A Survey Of Molecular Cloning Vectors And Their Uses* (Rodrigues and Denhardt, eds., Butterworths, 1987); Miller, *Experiments In Molecular Genetics* (Cold Spring Harbor Laboratory, 1972); and Miller, *A Short Course In Bacterial Genetics* (Cold Spring Harbor Laboratory, 1992).

As used herein, "vector" refers to an autonomously replicating nucleic acid unit. Many types of vectors are known with the most common and useful types being plasmid vectors, viral vectors, cosmid vectors, and phasmid vectors.

A diversity of vectors possessing different promoters, multiple cloning sequences, and different plasmid replicons can be used, so that the amount of a synthesized foreign antigen can be controlled by the relative number of gene copies. For example, vectors with p15A, pBR and pUC replicons can be constructed, all of which are dependent on the *polA* gene encoding DNA polymerase I for their replication. Preferably, vectors used in the Environmentally Limited Viability System do not use antibiotic resistance to select for maintenance of the vector.

A preferred vector, pMEG-104, including both an essential gene and lethal gene is shown in Figures 4. This vector is suitable for the expression of various foreign antigens in a recombinant avirulent *Salmonella*,

and in other enteric bacteria modified by introduction of a defined *asd* deletion containing the repressor elements by using the suicide vector pMEG-096 (Figure 3). Genes for such antigens can be cloned into the multiple cloning site and expressed under the control of the constitutive *P_{trc}*. The host strain has the λ *cI857* gene chromosomally located and constitutively expressed under the control of the native promoter (P_{RM}). The λ *cI857* gene product is inactive at 37°C and will not repress the λP_L which is driving the expression of the chromosomal P22 *c2* wild-type gene (the *c2* gene in P22 is equivalent to the *cI* gene in lambda). The expression of the P22 *c2* gene at 37°C will repress the P22 P_R and thus preclude expression of the plasmid-encoded lysis genes 13 and 19. The *asd* gene in the plasmid vector will be expressed, however, since the λ *cI857* protein is inactive at 37°C and the *asd* gene will be expressed under the control of λP_L . At 30°C or lower, the λ *cI857* protein exhibits a wild-type phenotype. It will repress the expression of the P22 *c2* gene, which will therefore result in an insufficient quantity of *c2* repressor and the lysis genes *lys13* and *lys19* will be expressed because of transcription initiated at the P22 P_R . On the other hand, the functional λ *cI857* gene product will now repress λP_L leading to cessation of synthesis of the *asd* gene product. It should be noted that the *asd* genes and the lysis genes *lys13* and *lys19* are arranged with no transcription terminations between them such that at 30°C and below, the mRNA for the lysis genes *lys13* and *lys19* will extend into the *asd* gene but with an incorrect orientation and thus constitute an antisense RNA that will further eliminate expression of the *asd* gene. Just the opposite occurs at 37°C such that the *asd* gene message will extend into the lysis genes 13 and 19 and serve as an antisense RNA to completely shut down the expression of the lysis genes. It should be noted that if the plasmid is transferred to another microorganism, the lysis genes will be expressed constitutively and lead to the demise of the recipient cell. Thus the containment host-vector system prevents both survival and

spread of the microbial strain within the environment and transmission of recombinant plasmids within natural populations of bacteria.

Preferred bacterial hosts/strains and vectors useful in, or useful for constructing, Environmentally Limited Viability Systems are listed in
5 Tables 1, 2, and 3.

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Table 1
Strains and Plasmids Useful in the Construction and Confirmation
of Environmentally Limited Viability Systems

| Strain | Description | Genotype |
|---------|---|--|
| χ1891 | <i>E. coli</i> K-12 temperature sensitive <i>polA</i> mutant | <i>F⁻ thr tss purE supE42 Δ⁻ trpE his gyrA srl thyA57 T3^r mtlA polA12_(his) cysA cycB</i> |
| χ3730 | <i>S. typhimurium</i> LT-2 Asd ⁻ intermediate host strain that is reversibly rough and has three of the <i>S. typhimurium</i> host restriction systems inactivated. | <i>leu hsdLT galE trpD2 tpsL120 asdA1 [zhf-4:Tn10] metE551 metA22 hsd5A hsdSB ilv</i> |
| χ3761 | <i>S. typhimurium</i> UK-1 virulent wild-type strain obtained as a chicken passaged spleen isolate. | wild-type |
| MGN-026 | <i>E. coli</i> K-12 DH5α isogenized with λpir from χ6151. This strain provides <i>lacZα</i> complementation and the ability to support <i>pir</i> -dependent replicons. This strain also transforms well, is an endonuclease mutant, and is recombination deficient, making it useful for recombinant work with the pGP704 derived suicide vectors. | <i>endA1 hsdR17 (rk⁻, mk⁺) supE44 thi-1 recA1 gyrA relA1 (lacZYA-argF) U169 λpir deoR (φ80dttac (lacZ)M15)</i> |
| MGN-336 | <i>E. coli</i> strain for the P22 lysis vectors obtained by electroporating χ6097 with pMEG-096 to provide a single integration of the <i>c2</i> repressor genes in the <i>asd</i> gene. This provides the λP _R driven P22 <i>c2</i> gene and lambda <i>cI857</i> repressor between either part of the <i>S. typhimurium asd</i> deletion inserted into the chromosome, duplicating part of the <i>asd</i> gene of <i>E. coli</i> with that of <i>S. typhimurium</i> . | <i>Δ⁺a (lac-pro) rpsL asdA4 [zhf-2::Tn10] asd-17::cI857PRc2 thi φ80dttacZ M15</i> |
| MGN-377 | <i>S. typhimurium</i> UK-1 intermediate ELVS host strain obtained by electroporating χ3761 with large amounts of pMEG-096 (Figure 3) to obtain a single integration of this <i>cI857PR c2</i> clone in the <i>asd</i> gene. This provides the λP _R driven P22 <i>c2</i> gene and λ <i>cI857</i> between either part of the <i>S. typhimurium asd</i> deletion duplicating portions of the wild-type <i>asd</i> gene in the chromosome. | wild-type + integrated pMEG-096 |

| | | |
|-----------------------------|---|--|
| MGN-391 | <i>S. typhimurium</i> UK-1 ELVS host obtained from MGN-377 by selecting for fusaric acid resistant, tetracycline sensitive, Asd ^r isolates produced by excision of pMEG096 in MGN-377 leaving the <i>cl857PRc2</i> cartridge in the defined <i>asd</i> deletion. Sibling of MGN-392 (Table 3). | <i>asd-17::cl857PRc2</i> |
| MGN-399 (pMEG-100 + pYA232) | <i>S. typhimurium</i> UK-1 ELVS host MGN-392 containing the intermediate ELVS vector pMEG-100 (Figure 5), and the <i>lacI^q</i> repressor plasmid pYA232 (Table 2). Obtained by electroporating MGN-392 with pMEG-100 and pYA232. Does not grow at temperatures below 30°C. | (pMEG-100 + pYA232) <i>asd-17::cl857PRc2</i> |
| MGN-401 (pYA292) | <i>S. typhimurium</i> UK-1 ELVS host MGN-392 electroporated with the Asd ^r vector pYA292 to provide a positive control for growth of this strain at different temperatures without DAP. | (pYA292) <i>asd-17::cl857PRc2</i> |
| MGN-409 | <i>E. coli</i> K-12 strain MGN-336 electroporated with ELVS vector pMEG-104 (Figure 4) This construct does not grow at temperatures below 30°C. | <i>catA (lac-pro) rpsL asdA4 [zhf-2::Tn10] asd-17::cl857PRc2 thi</i> <i>φ80lacZDM15</i> |

Table 2
Plasmids Useful in the Construction and Confirmation
of Environmentally Limited Viability Systems

| Plasmid | Description | Genotype |
|----------|---|----------|
| pMEG-011 | A <i>pir</i> -dependent suicide vector with <i>lacZα</i> multiple cloning site derived from pGP704. | |
| pMEG-072 | The promoter right of P22HTint, PCR amplified and cloned as a 125 bp <i>Bam</i> HI- <i>Sa</i> I fragment into the <i>Bam</i> HI- <i>Sa</i> I sites of pKK232-8 | |
| pMEG-076 | The promoter left of lambda PCR amplified from the lambda lysogen x2869 and cloned as a 163 bp fragment into the <i>Sma</i> I- <i>Bam</i> HI sites of pKK232-8 | |
| pMEG-078 | The P22HTint lysis genes <i>lys13</i> and <i>lys19</i> PCR amplified as a 797 bp fragment with a SD site at a 5' blunt end and a <i>Pst</i> I site designed into the 3' end, cloned into the <i>Eco</i> RV- <i>Pst</i> I sites of the low copy vector pWKS30 | |
| pMEG-086 | An Asd ⁺ clone obtained by inserting the 314 to 1421 bp <i>asd</i> PCR product lacking the promoter and SD regions as a 1115 bp <i>Bgl</i> II - blunt fragment, into the <i>Bam</i> HI- <i>Pvu</i> II sites of pMEG-076. This places <i>asd</i> under the regulation of λP_L | |
| pMEG-088 | A PCR clone of the P22 <i>c2</i> gene driven by λP_R and controlled by $\lambda cI857$. | |
| pMEG-089 | The 1060 bp <i>Sa</i> II- <i>Pvu</i> II fragment of pMEG-078 containing the P22 lysis genes <i>lys13</i> and <i>lys19</i> driven by the P22 promoter right cloned into the <i>Sa</i> II- <i>Pvu</i> II sites of pMEG-072. This construct is toxic unless in a strain expressing P22 <i>c2</i> repressor | |
| pMEG-090 | Intermediate expression vector obtained by <i>Bgl</i> II partial digestion of pYA810, isolating the 1.6 kb fragment containing the p15A origin of replication, <i>Pirc</i> , multiple cloning site and 5ST1T2, and ligating to the 1.3 kb <i>Bam</i> HI Km ^r cartridge of pUC-4K | |

| | | |
|----------|---|--|
| pMEG-096 | The ~2.05 kb <i>ClaI</i> fragment of pMEG-088 ligated into the <i>BglIII</i> site of pMEG-006 following treatment with T4 DNA polymerase. This provides the λP_R driven P22 <i>c2</i> gene and <i>cl857</i> between either part of the <i>S. typhimurium asd</i> deletion allowing for chromosomal insertion. | |
| pMEG-097 | The 1277 bp <i>EcoRI-XbaI</i> fragment of pMEG-86 containing the $\lambda P_{L}asd$ gene cloned into the <i>PvuII-XbaI</i> sites of pMEG-89 using the <i>trpA</i> terminator linker with an internal <i>BglII</i> site and an <i>EcoRI</i> end. This construct is lethal if not in a host with a P22 <i>c2</i> repressor. | |
| pMEG-098 | Derived from pMEG-097 by removing the 28 bp <i>PstI-XbaI</i> fragment of pMEG-097 treating with T4 DNA polymerase, and religating. | |
| pMEG-100 | ELVS expression vector intermediate obtained by ligating the 2.26 kb <i>BamHI-BglIII</i> fragment of pMEG-098 containing the <i>asd</i> gene into the <i>BglIII</i> site of pMEG-090. | |
| pMEG-104 | ELVS p15A expression vector obtained by deleting the 1.36 kb <i>Sall</i> fragment of pMEG-100 containing the <i>Km'</i> cartridge. Requires host with P22 <i>c2</i> repressor. | |
| pYA232 | Tc ^r , <i>lacI^H</i> repressor on pSC101 vector | |
| pYA248 | p15A <i>Asd⁺</i> expression vector, using <i>S. mutans asd</i> gene (Nakayama <i>et al.</i> 1988). | |
| pYA292 | p15A <i>Asd⁺</i> expression vector, using <i>S. typhimurium asd</i> gene (Galan <i>et al.</i> 1990). | |
| pYA810 | p15A <i>Asd⁺</i> expression vector, using <i>S. typhimurium asd</i> gene. Derived from pYA292 by removing the <i>HindIII</i> fragment of the multiple cloning site containing the <i>lacZα</i> gene. | |

F. Microbial Hosts

Any bacteria in which an essential gene is known for which mutants can be made, or for which a lethal gene is available, can serve as the host for an Environmentally Limited Viability System. Generally, those bacteria that are normally found in a target environment would be used in an Environmentally Limited Viability System targeted to that environment. It is preferred that promoters and regulatory elements of the system be native for the host being used, but this is not required. For example, many bacterial promoters are functional in heterologous hosts. Preferred host cells useful in, or useful for constructing, Environmentally Limited Viability Systems are listed in Tables 1 and 3.

1. **Obtaining defective mutants.** Defective mutants can be obtained as described above. For example, deletion mutations in the *asd* gene (Δasd) can be made in a diversity of bacterial strains that are members of the *Enterobacteriaceae*, and in other gram-negative bacteria and mycobacteria. *E. coli* K-12 and *S. typhimurium* LT-2 strains can be used to isolate *asd* mutants and their derivatives; the Asd^- strains can be used to construct other strains, utilizing transposon techniques, as described infra. Asd^- strains are also described in U.S. Patent No. 4,190,495.

Standard mutagenesis and mutant enrichment protocols are not efficient for the recovery of *asd* mutants, since a mutant with a requirement for DAP undergoes lysis and death in the absence of DAP. In a synthetic medium, *asd* mutants require L-methionine, L-threonine, and DAP for growth. The requirement for L-methionine and L-threonine is satisfied by homoserine, which is a common precursor to both methionine and threonine. Mutagenesis of an *E. coli* or *S. typhimurium* strain followed by an ampicillin-cycloserine procedure for the enrichment of auxotrophic mutants seldom, if ever, recovers mutants with a sole requirement for homoserine. Curtiss *et al.* (1965) describe a cycloserine-enrichment procedure for selecting

auxotrophs. The reason that homoserine-requiring auxotrophs are seldom isolated is that β -aspartic semialdehyde is converted to homoserine by either of two dehydrogenases which are encoded in two genes. The probability of inactivating both genes in a single cell is exceedingly small, and thus the

5 homoserine-requiring auxotrophs may not be detected by random screening techniques.

This problem is overcome by the inclusion of DAP in all media during mutagenesis, and by enrichment or selection using the ampicillin-cycloserine technique. This leads to the recovery of *asd* mutants that require

10 both homoserine and DAP. Ampicillin and cycloserine both inhibit cell wall synthesis in growing cells capable of protein synthesis, but are without effect on auxotrophic mutants unable to synthesize proteins because of the absence of nutritional requirements. *asd* mutant strains Chi3008 and Chi2108, which are *S. typhimurium* and *E. coli* strains, respectively, were isolated using this

15 procedure. The *Asd*⁻ phenotype of Chi3008 is due to a point mutation in the *asd* gene, and thus the frequency reversion to *Asd*⁺ is fairly high. On the other hand, the *Asd*⁻ phenotype of Chi2108 results from a deletion in the *asd* gene, thus, the reversion frequency is very low.

Deletion mutations containing insertions of foreign genes

20 encoding regulatory elements for an ELVS can be introduced into the bacterial chromosome using well known recombinant DNA techniques. For example, the gene to be deleted should first be cloned onto a suicide vector such as pMEG-011 (Figure 2). pMEG-011 is a *pir*-dependent suicide vector derived from pGP704 (Miller *et al.*, *J. Bacteriol.* 170:2575-2583 (1988)).

25 The replication of such a suicide vector is dependent on the production of a functional *pir* gene product by the host strain. When the suicide vector is transferred to a strain lacking the *pir* gene, the only means of maintaining the suicide vector is by recombination integrating the suicide vector into the host chromosome. The presence of the gene to be deleted on the suicide vector

results in homologous recombination of the suicide vector into the corresponding gene on the chromosome. This integration will result in a deletion in the gene of interest if the wild-type gene on the suicide vector has been altered by deletion of internal regions of the gene prior to integration.

5 This can be accomplished by either restriction enzyme digestion or inverse PCR amplification. The vector-borne gene of interest is thus inactivated while leaving sufficient flanking DNA to allow recombination into the chromosome. The defined deletion produced in the suicide vector can be designed to provide a convenient restriction enzyme cloning site allowing the
10 insertion of any foreign gene, including the regulatory genes needed for the Environmentally Limited Viability System. After the initial single recombination event integrating the suicide vector into the chromosome, a second recombinational event can be selected for by selection against the tetracycline element of the suicide vector on media containing fusaric acid
15 (Bochner *et al.*, *J. Bacteriol.* 143:926 (1980)). This results in the replacement of the wild-type allele with the deleted gene containing the desired insert. These methods have been used to produce defined deletions and insertion of foreign genes into the chromosome of *Salmonella* strains (Chatfield *et al.*, *Vaccine* 10:53-60 (1992)).

20 Although the preferred embodiment of the disclosed ELVS is a live bacterial vaccine, the ELVS can be used with any microbe in which ELVS components, as described above, can be obtained and adapted to that microbe. In particular, an ELVS will be useful when used in conjunction with the production of valuable products in fermenters, especially products
25 that might be toxic or otherwise harmful if the bacteria producing them escaped and survived.

2. Live bacterial vaccines. Preferred hosts for use as vaccines are enteric bacteria. As used herein, enteric bacteria refers to any *Enterobacteraceae*. Many of the preferred genes and regulatory elements

described above are operable in most enteric bacteria, thus allowing use of the many well developed *E. coli* and *Salmonella* regulatory systems. Most preferably, the bacterial host is an avirulent *Salmonella*.

5 In one embodiment of the system described herein, an avirulent derivative of a pathogenic microbe that attaches to, invades and persists in the gut-associated lymphoid tissue (GALT) or bronchial-associated lymphoid tissue (BALT) is used as a carrier of the gene product which is used for stimulating immune responses against a pathogen or allergen. Avirulent does not mean that a microbe of that genus or species can not ever function as a pathogen, but that the particular microbe being used is avirulent with respect to the particular animal being treated. The microbe may belong to a genus or even a species that is normally pathogenic but must belong to a strain that is avirulent. By pathogenic is meant capable of causing disease or impairing normal physiological functioning. Avirulent strains are incapable of inducing a full suite of symptoms of the disease that is normally associated with its virulent pathogenic counterpart. Microbes as used herein include bacteria, protozoa, and unicellular fungi.

Avirulent *Salmonella* containing an ELVS can be used to protect animals and humans against *Salmonella* infection. To be useful, such strains need not express any foreign antigen. Recombinant avirulent *Salmonella* vaccines expressing protective antigens from bacterial, viral, mycotic and parasitic pathogens that are efficacious in inducing immunities to protect against infections by these pathogens will be more acceptable and receive more widespread use if rendered safer by biological containment properties that preclude survival and proliferation in the environment. The developments proposed should potentiate the commercial value of vaccine technology based on the use of live bacterial vaccine vectors.

3. Avirulent Bacterial Hosts. To be useful as a live recombinant vaccine, the bacterial host must be avirulent. Strains of

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different *Salmonella* serotypes can be rendered avirulent and immunogenic by methods known to those skilled in the art, for example, 1) by introducing mutations that impose a requirement for aromatic amino acids and vitamins derived from precursors in this pathway (Stocker *et al.* (1983), Hoiseth and Stocker (1981)), 2) by deleting genes for global regulators such as *cya* and *crp* (Curtiss and Kelly (1987)), *phoP* (Miller *et al.*, *Proc. Natl. Acad. Sci. USA* 86:5054-8 (1989), Galan and Curtiss, *Microb. Pathogen.* 6:433-443 (1989)), and *ompR* (Dorman *et al.*, *Infect. Immun.* 57:2136-40 (1989)), 3) by deleting genes for lipopolysaccharide (LPS) synthesis, such as by *galE* (Germanier and Furer, *Infect. Immun.* 4:663-73 (1971), Germanier and Furer, *J. Infect. Dis.* 131:553-8 (1975)), although this alone may be insufficient (Hone *et al.*, *Infect. Immun.* 56:1326-1333 (1988)), 4) by mutating genes needed for colonization of deep tissues, such as *cdt* (Kelly *et al.*, *Infect. Immun.* 60:4881-4890 (1992); Curtiss *et al.*, *Devel. Biol. Stand.* 82:23-33 (1994)), or 5) by preventing expression of genes for proteases required at high temperature, such as *htrA* (Johnson *et al.*, *Mol. Microbiol.* 5:401-407 (1991). Strains possessing mutations in *phoQ* (Miller *et al.*, *Proc. Natl. Acad. Sci. USA* 86:5054 (1989)) have the same phenotype as mutations in *phoP*. Hereinafter strains with mutations in either *phoP* or *phoQ* are referred to collectively as *phoP* mutants. It is preferred that mutations in the above described genes be introduced as deletions since this will preclude reversion mutations and enhance the safety of vaccine strains containing them. Subsequent to the discovery that *Salmonella* strains with mutations in the genes described above are avirulent and immunogenic, it was observed that many of these strains exhibited, after oral administration, nearly wild-type abilities to invade and persist in the GALT and to colonize other lymphoid tissues such as mesenteric lymph nodes, liver, and spleen, but without causing disease symptoms. As a consequence, these attenuated vaccine strains are capable of stimulating strong mucosal, systemic and

cellular immune responses in immunized animal hosts that confer protective immunity to challenge with virulent wild-type *Salmonella* strains. Any of these avirulent *Salmonella* can be endowed with the ability to express important colonization or virulence antigens from other bacterial, viral, mycotic and parasitic pathogens at a high level within an immunized animal host (Clements, *Pathol. Immunopathol. Res.* 6:137-146 (1987); Dougan *et al.*, *Parasite Immun.* 9:151-60 (1987); Chatfield *et al.*, *FEMS Immunol. Med. Microbiol.* 7:1-7 (1993); Curtiss *et al.*, in *Virulence mechanisms of bacterial pathogens*, (Roth, American Society for Microbiology, Washington, D.C., 1988) pages 311-328; Curtiss *et al.*, *Dev. Biol. Stand.* 82:23-33 (1994); Doggett and Curtiss, *Adv. Exp. Med. Biol.* 327:165-73 (1992); Schödel, *Semin Immunol.* 2:341-9 (1990)).

G. Expression Genes

One of the primary uses of the disclosed Environmentally Limited Viability System is to allow recombinant expression of a desired expression product limited to a specific permissive environment. As used herein, expression gene refers to a gene expressed in an ELVS that encodes a desired expression product. Expression of a gene means that the information inherent in the structure of the gene (the sequence of DNA bases) is transformed into a physical product in the form of an RNA molecule, polypeptide or other biological molecule by the biochemical mechanisms of the cell in which the gene is located. The biological molecule so produced is called the expression product. The term expression product as used here refers to any biological product or products produced as a result of the biochemical reactions that occur under the control of a gene. The expression product may be, for example, an RNA molecule, a peptide, or a product produced under the control of an enzyme or other molecule that is the initial product of the gene, that is, a metabolic product. For example, a gene may first control the synthesis of an RNA molecule which is translated by the

action of ribosomes into an enzyme which controls the formation of glycans in the environment external to the original cell in which the gene was found. The RNA molecule, the enzyme, and the glycan are all expression products as the term is used here. Such expression products need not be heterologous to the host cell nor encoded by a recombinant gene. It is preferred, however, that the expression gene is recombinant. Most preferably, the expression gene encodes an antigen.

Expression of the expression gene can be accomplished, for example, by inserting the coding sequence encoding a desired gene product in the multiple cloning site of the ELVS vector pMEG-104 (Figure 4). This places the coding sequence of the expression gene under control of the constitutive promoter *P_{trc}*, with transcription terminating at *rrnB* thus preventing inappropriate transcription of the lysis genes.

Vaccine Antigens.

Live recombinant vaccines using an Environmentally Limited Viability System can be used to deliver any antigen that can be expressed in the host microorganism. For example, antigens can be from bacterial, viral, mycotic and parasitic pathogens, to protect against bacterial, viral, mycotic, and parasitic infections, respectively; gametes, provided they are gamete specific, to block fertilization; and tumor antigens, to halt cancers. The selection and recombinant expression of antigens has been previously described by Schödel (1992) and Curtiss (1990). Immunogenicity of the vaccines can be augmented and/or modulated by constructing strains that also express genes for cytokines.

Some examples are vaccines for the control of plague caused by *Yersinia pestis*, of gonorrhea caused by *Neisseria gonorrhoea*, of syphilis caused by *Treponema pallidum*, and of venereal diseases as well as eye infections caused by *Chlamydia trachomatis*. Species of *Streptococcus* from both group A and group B, such as those species that cause sore throat or

heart diseases, *Neisseria meningitidis*, *Mycoplasma pneumoniae*, *Hemophilus influenza*, *Bordetella pertussis*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Bordetella avium*, *Escherichia coli*, *Streptococcus equi*, *Streptococcus pneumoniae*, *Brucella abortus*, *Pasteurella hemolytica*, *Vibrio cholera*,

5 *Shigella* species, and *Legionella pneumophila* are additional examples of bacteria within the scope of this invention from which genes could be obtained. Viral antigens can also be used with the Environmentally Limited Viability System. Viral antigens can be used in vaccines directed against viruses, either DNA or RNA viruses, for example from the classes

10 *Papovavirus*, *Adenovirus*, *Herpesvirus*, *Poxvirus*, *Parvovirus*, *Reovirus*, *Picornavirus*, *Myxovirus*, *Paramyxovirus*, or *Retrovirus*. Vaccines using antigens of pathogenic fungi, protozoa and parasites can also be used.

The antigen can also be an allergen of the host such as antigens from pollen and animal dander. Such an antigen can be used in the Environmentally Limited Viability System in an exposure regimen designed to specifically desensitize an allergic host.

Recombinant avirulent *Salmonella* vaccines are capable of stimulating strong mucosal, systemic and cellular immune responses against the foreign antigens and thus against the pathogen that is the source of the foreign antigen. It is not necessary that the antigen gene be a complete gene as present in the parent organism, which was capable of producing or regulating the production of a macromolecule, for example, a functioning polypeptide. It is only necessary that the gene be capable of serving as the template used as a guide in the production of an antigenic product. The product may be one that was not found in that exact form in the parent organism. For example, a functional gene coding for a polypeptide antigen comprising 100 amino acid residues may be transferred in part into a carrier microbe so that a peptide comprising only 75, or even 10, amino acid residues is produced by the cellular mechanism of the host cell.

Alternatively, if the amino acid sequence of a particular antigen or fragment thereof is known, it is possible to chemically synthesize the DNA fragment or analog thereof by means of automated gene synthesizers or the like and introduce said DNA sequence into the appropriate expression vector. At the
5 other end of the spectrum is a long section of DNA coding for several gene products, one or all of which can be antigenic. Thus a gene as defined and claimed here is any unit of heredity capable of producing an antigen. The gene may be of chromosomal, plasmid, or viral origin.

Multiple antigens can also be expressed by a recombinant
10 avirulent *Salmonella* vaccine. In addition, antigens, or even parts of antigens, that constitute a B cell epitope or define a region of an antigen to which an immune response is desired, can be expressed as a fusion to a carrier protein that contains a strong promiscuous T cell epitope and/or serves as an adjuvant and/or facilitates presentation of the antigen to enhance,
15 in all cases, the immune response to the antigen or its component part. This can easily be accomplished by genetically engineering DNA sequences to specify such fusions for expression by avirulent vaccine strains. Fusion to tetanus toxin fragment C, CT-B, LT-B and hepatitis virus B core are particularly useful for these purposes, although other epitope presentation
20 systems are well known in the art.

In order for the expression gene to be effective in eliciting an immune response, the expression gene must be expressed, which can be accomplished as described above. In order for a vaccine to be effective in immunizing an individual, the antigenic material must be released in such a
25 way that the immune system of the vaccinated animal can come into play. Therefore the live avirulent microorganism must be introduced into the animal. In order to stimulate a preferred response of the GALT or BALT cells as discussed previously, introduction of the microbe or gene product directly into the gut or bronchus is preferred, such as by oral administration,
30 gastric intubation or in the form of aerosols, although other methods of

administering the vaccine, such as intravenous, intramuscular, subcutaneous injection or intramammary or intrapenial or vaginal administration, is possible.

H. Vaccine Compositions

5 Oral immunization in a suitable animal host with live recombinant *Salmonella* vaccine strains leads to colonization of the gut-associated lymphoid tissue (GALT) or Peyer's patches, which leads to the induction of a generalized mucosal immune response to both *Salmonella* antigens and the foreign antigens synthesized by the recombinant *Salmonella* (Curtiss *et al.*, 10 *Adv. Exp. Med. Biol.* 251:33-47 (1989)). Further penetration of the vaccine strain into the mesenteric lymph nodes, liver and spleen augments the induction of systemic and cellular immune responses directed against *Salmonella* antigens and the foreign antigens made by the recombinant *Salmonella* (Doggett and Curtiss (1992)). Thus the use of recombinant 15 avirulent *Salmonella* vaccines for oral immunization stimulates all three branches of the immune system, particularly important when immunizing against infectious disease agents which colonize on and/or invade through mucosal surfaces.

By vaccine is meant an agent used to stimulate the immune 20 system of a living organism so that an immune response occurs. Preferably, the vaccine is sufficient to stimulate the immune system of a living organism so that protection against future harm is provided. Immunization refers to the process of inducing a continuing high level of antibody and/or cellular immune response in which T- lymphocytes can either kill the pathogen 25 and/or activate other cells (for example, phagocytes) to do so in an organism, which is directed against a pathogen or antigen to which the organism has been previously exposed. Although the phrase "immune system" can encompass responses of unicellular organisms to the presence of foreign bodies, that is, interferon production, as used herein the phrase is restricted

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administration could be 1×10^7 to 1×10^{11} CFU depending upon the size and age of the individual to be immunized. Administering multiple dosages can also be used as needed to provide the desired level of protective immunity.

- 5 The pharmaceutical carrier in which the vaccine is suspended can be any solvent or solid material for encapsulation that is non-toxic to the inoculated animal and compatible with the carrier organism or antigenic gene product. Suitable pharmaceutical carriers include liquid carriers, such as normal saline and other non-toxic salts at or near physiological concentrations, and solid carriers not used for humans, such as talc or sucrose, or animal feed.
- 10 Adjuvants may be added to enhance the antigenicity if desired. When used for administering via the bronchial tubes, the vaccine is preferably presented in the form of an aerosol.

- Immunization with a pathogen derived gene product can also be used in conjunction with prior immunization with the avirulent derivative of a pathogenic microorganism acting as a carrier to express the gene product specified by a recombinant gene from a pathogen. Such parenteral immunization can serve as a booster to enhance expression of the secretory immune response once the secretory immune system to that pathogen-derived gene product has been primed by immunization with the carrier microbe
- 15 expressing the pathogen derived gene product to stimulate the lymphoid cells of the GALT or BALT. The enhanced response is known as a secondary, booster, or anamnestic response and results in prolonged immune protection of the host. Booster immunizations may be repeated numerous times with beneficial results.

25 I. Adaptation of Environmentally Limited Viability Systems to Useful Host Strains.

- Avirulent strains of *S. typhimurium* are known to be totally attenuated and highly immunogenic in mice, chickens, and pigs, inducing protective immunity to infection with 10,000 times a lethal dose with the virulent wild-type strain. Similarly, avirulent strains of *S. choleraesuis* are
- 30

Bacterial pathogens can be attenuated by introducing deletion (Δ) mutations in various genes as described above or as known to those knowledgeable in the art. Any of these strains are suitable for introduction of an ELVS of the sort disclosed herein, although modifications would be needed to make the system operable in gram-positive bacteria. Specifically these modifications would require modification of Shine-Dalgarno sequences to permit translation of mRNA, slight changes in promoter sequences to cause transcription to be more efficient, and, in the case of all gram-positive

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genera other than *Mycobacteria*, the *asd* gene would need to be replaced by some other essential gene. This is because DAP is not contained in the rigid layer of the cell wall of most gram-positive bacterial genera except for the *Mycobacteria*. On the other hand, there are numerous essential genes for

5 essential components of the cell wall, cell membranes, and for maintaining the integrity of DNA that could be used in both gram-positive and gram-negative bacteria. The components of the ELVS described in Figure 4 can readily be introduced into *Salmonella* species, *E. coli*, *Shigella* species and other enterics. For example the suicide vector pMEG-096, can be

10 transferred from a *pir*-containing donor strain to an attenuated vaccine candidate with a wild-type *asd* gene. Integration of the *cl857PRc2* cartridge of pMEG-096 into the candidate strain can be accomplished by first selecting tetracycline resistant clones that have integrated pMEG-096, and then growing the cells on fursaric acid-containing media to select for cells that

15 have lost the wild-type *asd* gene in favor of the *asd* defined deletion containing the *cl857PRc2* cartridge. These manipulations are described in the examples below. A derivative of pMEG-104 (Figure 4) containing a gene encoding a desired foreign antigen, could then be introduced by electroporation. The resulting recombinant avirulent vaccine with its ELVS

20 can then be used to immunize suitable hosts. Excretion or shedding of any live vaccine cells by the vaccinated hosts will activate the ELVS which will lead to death by lysis of these vaccine cells. This will preclude the survival of the vaccine outside of the host animal and thus eliminate the possibility for immunization of individual hosts that did not elect to be immunized.

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Deposits of Strains Useful in Practicing the Invention.

A deposit of biologically pure cultures of the following strains (Table 3) were made with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland. The accession number indicated was

assigned after successful viability testing, and the requisite fees were paid. Access to the cultures will be available during pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 USC 122. All restriction on availability of the

5 cultures to the public will be irrevocably removed upon the granting of a patent based upon the application. Moreover, the designated deposits will be maintained for a period of thirty (30) years from the date of deposit, or for

10 five (5) years after the last request for the deposit; or for the enforceable life of the U.S. patent, whichever is longer. Should a culture become nonviable or be inadvertently destroyed, or, in the case of plasmid-containing strains, loose its plasmid, it will be replaced with a viable culture(s) of the same taxonomic description.

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Table 3
Strains Deposited with ATCC for Implementation of Environmentally Limited Viability System

| Strain (plasmid) | ATCC No. | Deposit Date | Description | Genotype |
|-----------------------|-------------|-----------------|---|--|
| MGN-318 (pMEG-096) | | 6/6/95 | <i>E. coli</i> MGN-026 containing pMEG-096. pMEG-096 (Figure 3) contains the λP_R driven P22 c2 repressor gene and $\lambda cI857$ repressor gene on a 2.05 kb <i>Clal</i> fragment from pMEG-088 treated with T4 DNA polymerase and ligated into the <i>Bgl</i> III site of the <i>asd</i> deletion vector pMEG-006 following treatment with T4 DNA polymerase and CIAP. This provides the λP_R driven P22 c2 gene and $\lambda cI857$ repressor gene between either end of the <i>S. typhimurium asd</i> deletion allowing insertion into the chromosome. | (pMEG-096 <i>asd-17::cI857PRc2</i>) <i>endA1 hsdR17 (rk-, mk+) supE44 thi-1 recA1 gyrA relA1 (lacZYA-argF) U169 λpir deoR</i> ($\phi 80d\text{tac}$ (<i>lacZ</i>)M15) |
| MGN-392 | | 6/6/95 | <i>S. typhimurium</i> UK-1 ELVS host obtained from MGN-377 by selecting for fusaric acid resistant, tetracycline sensitive, <i>Asd</i> ⁻ isolates produced by excision of pMEG-096 in MGN-377 leaving the <i>cI857PRc2</i> cartridge in the defined <i>asd</i> deletion. | <i>asd-17::cI857PRc2</i> |
| MGN-417 (pMEG-104) | | 6/6/95 | <i>S. typhimurium</i> UK-1 ELVS host MGN-392 electroporated with the ELVS expression vector, pMEG-104 (Figure 4), an <i>asd</i> lysis p15A expression vector obtained by deleting the 1.36 kb <i>Sal</i> I fragment of pMEG-100 (Table 2, Figure 5) containing the <i>Km</i> ^r cartridge. This construct does not grow at temperatures below 30°C. Plasmid requires host with functional P22 c2 gene. | (pMEG-104 P22P _R <i>lys13 lys19 asd λP_L</i>) <i>asd-17::cI857PRc2</i> |

Described below are examples of the present invention which are provided only for illustrative purposes, and not to limit the scope of the present invention. In light of the present disclosure, numerous embodiments within the scope of the claims will be apparent to those of ordinary skill in the art.

Examples

Example 1: Construction of a plasmid based thermoregulated Environmentally Limited Viability System.

The methods used to produce the components of the ELVS illustrated in Figure 4 employed standard molecular cloning techniques. The ELVS depicted in Figure 4 consists of two main components. The first component is the ELVS vector which combines the application of a temperature regulated essential gene, *asd*, with the temperature regulated lethal genes, *lys13* and *lys19*, oriented in a manner to provide additional regulation by antisense RNA for both essential and lethal genes. The second component is the ELVS host required to maintain the ELVS vector, which for the *S. typhimurium* examples used the host MGN-392, possessing a defined *asd* deletion with a thermosensitive *cl857* and *c2* repressor cartridge insert.

The ELVS expression vector pMEG-104, was constructed by the assembly of six individual components consisting of 1) the *S. typhimurium asd* gene, 2) the bacteriophage lambda promoter left to drive the *asd* gene, 3) the bacteriophage P22 lysis genes *lys13* and *lys19*, 4) the bacteriophage P22 promoter right to drive the lysis genes, 5) the *trpA* terminator to prevent transcription from external promoters, and 6) the expression vector core obtained from pYA810 (Galan *et al.* (1990)), consisting of the p15A origin of replication, the strong *trc* promoter, multiple cloning site used in existing *Asd*⁺ vectors, and the ribosomal transcription terminators 5S T1 T2 to

prevent transcriptional interference from the *trc* promoter. Many of these vector components were obtained by PCR amplification as described below, followed by modification of the DNA ends with either restriction enzymes or polymerases to allow assembly of the intermediate constructs. The *asd* gene of *S. typhimurium* was obtained by PCR amplification of the region between nucleotides 314 to 1421 of the *S. typhimurium asd* sequence as found on pYA292 (Galan *et al.* (1990)). This *asd* PCR product, a 1115 bp fragment with one end blunt and the other having a *Bgl*III overhang and lacking the promoter and Shine-Dalgarno regions of the *asd* gene, was cloned into the lambda promoter left vector, pMEG-076, in an orientation to produce an *asd* gene driven by the bacteriophage lambda promoter left. The result is plasmid pMEG-086.

The promoter left of bacteriophage lambda present on pMEG-076 was obtained by PCR amplification of the region between nucleotides 5 to 160 of the promoter left sequence (Giladi *et al.*, *J. Mol. Biol.* 231:109-121 (1990)). The resulting 155 bp fragment, with a blunt end and a *Bam*HI end, was cloned into the *Sma*I to *Bam*HI sites of the promoter probe cloning vector pKK232-8 (Brosius, *J. Gene* 27:151 (1984)), resulting in vector pMEG-086. In this vector, the *asd* gene is driven by the lambda promoter left.

The 1277 bp *Eco*RI to *Xba*I fragment of pMEG-086 containing the λP_L driven *asd* gene was then sub-cloned into the *Pvu*II to *Xba*I sites of the bacteriophage P22 *PR lys13 lys19* vector pMEG-089 using a synthetic *trpA* terminator, containing an internal *Bgl*III site and an *Eco*RI end, to produce pMEG-097. The relative orientation of the transcriptional elements for *asd* and *lys13 lys19* in pMEG-097 results in convergent transcription which produces antisense RNA for the *asd* and *lys* genes at differential temperatures.

The bacteriophage P22 lysis genes used in the construction of pMEG-097 were obtained by PCR amplification of nucleotides 62 to 850 of the P22 lysis gene region (Rennell *et al.*, *Virology* 142:280-289 (1985)). The resulting 797 bp fragment, with an introduced Shine-Dalgarno site, a 5' blunt end, and a *Pst*I site designed into the 3' end, was cloned into the *Eco*RV to *Pst*I sites of the low copy-number vector pWKS30 (Kushner, S. *Gene* 100:195-199 (1990)). The resulting vector, pMEG-078, has the *lys*13 and *lys*19 genes under control of the *lac* promoter. The lysis genes were then sub-cloned as a 1060 bp *Sal*I to *Pvu*II fragment from pMEG-078 into the *Sal*I to *Pvu*II sites of the P22 promoter right expression vector, pMEG-072. In the resulting vector, pMEG-089, the bacteriophage P22 lysis genes *lys*13 *lys*19 are driven by the P22 promoter right. The bacteriophage P22 promoter right in pMEG-072 was obtained by PCR amplification of the region between nucleotides 23 and 138 of the P22 promoter right sequence (Poteete *et al.*, *J. Mol. Biol.* 137:81-91 (1980)). The resulting 125 bp *Bam*HI to *Sal*I fragment was cloned into the *Bam*HI to *Sal*I sites of the promoter probe vector pKK232-8 (Brosius, J., *Gene* 27:151 (1984)) to produce pMEG-072.

The *asd lys*13 *lys*19 cartridge present in pMEG-097 contains both the essential and lethal genes separated by a portion of the multiple cloning site, including a *Bam*HI site from previous manipulations. This region was deleted from pMEG-097 as a 28 bp *Pst*I-*Xba*I fragment, and the remaining vector was treated with T4 DNA polymerase prior to religating. This resulted in vector pMEG-098, containing the *asd lys*13 *lys*19 cartridge. The *asd lys*13 *lys*19 cartridge was then transferred to the expression vector core of pMEG-090 by digesting pMEG-090 with *Bgl*II and inserting the 2.26 kb *Bam*HI to *Bgl*II fragment of pMEG-098 containing the *asd lys*13 *lys*19 cartridge. This produced pMEG-100, shown in Figure 5.

Vector pMEG-090 was derived from pYA810 by performing a partial *Bgl*II digest of pYA810; isolating the 1.6 kb fragment of pYA810

containing the p15A origin of replication, the *trc* promoter, and the multiple cloning site with the transcriptional terminator; and ligating the fragment to the 1.3 kb *Bam*HI kanamycin resistance element of pUC-4K (Christie G.E., *et al.*, *Proc. Natl. Acad. Sci. USA* 78:4180 (1981)).

5 In pMEG-100, the essential and lethal genes are flanked by two different transcriptional terminators, the first is the *trpA* terminator (Christie *et al.*, *Proc. Natl. Acad. Sci. USA* 78:4180 (1981)) and the other is the ribosomal 5S terminator repeat cartridge used in the previously described Asd⁺ balanced-lethal vectors pYA248 and pYA292 (EP 89900028.5). Vector
10 pMEG-100 also contains the lower copy-number DNA polymerase I-dependent p15A origin of replication and the strong *trc* promoter derived from pYA810 and used in the pYA292 Asd⁺ vector described in EP 89900028.5. The kanamycin resistance element of pMEG-100 was then removed by a partial *Sa*II digest to produce pMEG-104, the ELVS vector
15 shown in Figure 4.

The second component of the ELVS is the host strain containing the regulator elements responsible for the response of the modified bacteria to the environmental conditions encountered. In the present example the host is MGN-392 or the sibling strain MGN-391, which contain a *cI857PRc2*
20 cartridge. The *cI857PRc2* cartridge contains the bacteriophage lambda promoter right driven bacteriophage P22 *c2* repressor gene and the bacteriophage lambda temperature sensitive repressor gene *cI857*. The cartridge is located between flanking portions of the *S. typhimurium asd* gene and replaces the chromosomal *asd* gene of the wild-type *S. typhimurium*
25 strain χ 3761. More specifically, the *cI857PRc2* cartridge is a combination of a PCR amplified fragment of bacteriophage P22 derived from nucleotides 1 to 650 of the *c2* gene (Sauer, R. *et al.*, *Biochem.* 20:3591-3598 (1981), and a PCR amplified fragment of bacteriophage lambda, from nucleotides 37219 to 38035 of the lambda genome (Sanger, F., *et al.*, *J. Mol. Biol.* 162:729-

773 (1982), which contains the *cI857* gene and the lambda promoter right. On the cartridge, the lambda promoter right drives expression of the P22 *c2* gene. This cartridge has been inserted between the flanking portions of a defined deletion of the *S. typhimurium asd* gene lacking the portion of the *asd* gene between nucleotides 243 and 1460. This deletion prevents production of any β -aspartate semialdehyde dehydrogenase by the host strain MGN-392.

The function of the ELVS is demonstrated for both *Salmonella* and *E. coli* using the host strains MGN-392 for *S. typhimurium* (Table 3) and MGN-336 for *E. coli* (Table 1). MGN-392 was electroporated with either the Asd^+ vector pYA292, to produce MGN-401 (Table 1), or the ELVS expression vector pMEG-104, to produce MGN-417 (Table 3). MGN-336 was electroporated with the ELVS expression vector pMEG-104, to produce MGN-409 (Table 1). These strains were then grown for 12 to 16 hours in Lennox broth without aeration at 37°C and dilutions plated onto Lennox agar followed by incubation at the temperature indicated for up to 72 hours. The plating efficiency, measured as colony forming units, observed at room temperature (25°C) for MGN-417 is approximately 10,000 less than that observed for MGN-401, containing the Asd^+ vector, at the same temperature. The colonies observed after room temperature incubation of *E. coli* strain MGN-409 containing the ELVS vector pMEG-104 were extremely small, indicating poor growth survival (Table 4). The reduction obtained in plating efficiency of MGN-417 at 25°C in the presence of DAP indicates that the lysis genes are functioning alone to cause greater than 95% killing.

Table 4
Plating Efficiency of Strains Containing Temperature
Regulated ELVS Vectors^a

| | | | | |
|----|---|--|--|--|
| 5 | Strain (plasmid) | L agar at 25°C CFU at 10 ⁻⁴ dil. | L agar at 37°C CFU at 10 ⁻⁷ dil. | L agar + DAP at 25°C CFU at 10 ⁻⁷ dil. |
| | <i>S. typhimurium</i> MGN-401 (pYA292) | > 10,000 | 23 | N/A |
| 10 | <i>S. typhimurium</i> MGN-417s (pMEG-104) | 1 mucoid | 67 | 1 mucoid |
| 15 | <i>E. coli</i> MGN-409e (pMEG-104) | ~ 10,000 very small | 36 | N/A |

^aColony forming units (CFU) of cultures diluted 10⁻⁴ or 10⁻⁷ plated on L agar or L agar + DAP, after 72 hrs incubation at designated temperature.

This reduction in viability is further demonstrated by the data in Figure 6 for the *Salmonella* based ELVS in which MGN-417 (Table 3) fails to grow in complex liquid media at room temperature. Under these conditions, MGN-417 is unable to proliferate and exhibits a reduction in recoverable bacteria over a 24 hour period at the non-permissive temperature of 25°C, while still growing as well as MGN-401 (Table 1), the ELVS host MGN-392 containing the Asd⁺ vector pYA292, at the permissive temperature of 37°C.

**Example 2: Restricted transfer of Environmentally Limited
Viability Vector DNA to Other Bacterial Hosts.**

This example demonstrates the inability of bacterial hosts not containing the *cI857PRc2* cartridge to maintain the Environmentally Limited Viability System vector, even at permissive temperatures. During the construction of pMEG-104, as described earlier, the vector pMEG-100, containing a kanamycin resistance gene, was produced (Figure 5). Vector pMEG-100 and the tetracycline resistance plasmid pYA232 were co-purified from MGN-399 (Table 1). Vector pYA232 has a low copy-number pSC101 replicon that is compatible with the p15A origin of replication present on pMEG-100. The pool of plasmid DNA isolated from MGN-399 was then electroporated into an Asd^r *S. typhimurium* host, χ 3730, lacking the *c2* repressor gene necessary for repression of the P22 lysis genes. In this strain, the lysis genes on pMEG-100 should limit the transformability by pMEG-100. The same pool of plasmid DNA was also electroporated into host strain MGN-391 (Table 1) to confirm the transformability of the DNA. The inability of the non-specific host, χ 3730, to propagate the ELVS plasmid is evidenced by an approximately 3,000-fold drop in the number of electroporants obtained with pMEG-100 relative to pYA232 within the same DNA mix (Table 5). The CFU on L agar with tetracycline and DAP reveal the frequency of recovery of pYA232.

Table 5

Restriction of Plasmid Transfer to Non-permissive Hosts^a(CFU at 10⁻¹ dilution)

| Strain | DNA | LA- | LA+Tet+DAP | LA+Km |
|-------------|----------------------|---------|------------|---------|
| χ 3730 | - | 0 | 0 | 0 |
| χ 3730 | pMEG-100 + pYA232 | 16 | >1,000 | 4 |
| MGN-391 | - | 0 | 0 | 0 |
| MGN-391 | pMEG-100 + pYA232 | >10,000 | ~1,000 | >10,000 |

^aa mixture of pMEG-100 and pYA232 plasmid DNAs were isolated from *S. typhimurium* MGN-399 and used to transform the bacterial strains listed by electroporation.

^bCFU (colony forming units) were determined by incubating dilutions spread on LA (Lennox agar) plates with or without tetracycline (Tet; 15 μ g/ml); diaminopimelic acid (DAP; 50 μ g/ml) or kanamycin (Km; 30 μ g/ml) at 37°C.

Example 3: Construction of a Temperature-Regulated *polA* Host.

This example describes the design of a Δasd Environmentally Limited Viability System in which the effectiveness of the Environmentally Limited Viability System is further ensured by employing a temperature regulated *polA* gene. The chromosomal *polA* gene has been altered to be expressed at 37°C but not at temperatures below 30°C, thus preventing further replication of *polA*-dependent replicons containing the essential *asd* gene at the non-permissive temperature. As described above, the replication of plasmids with the p15A, pBR and pUC origins of replication are dependent on DNA polymerase I. These replicons are widely used in cloning vectors. The inability of these *polA*-dependent plasmids to replicate at

temperatures below 30°C will then result in plasmid vector loss from a population of cells growing at temperatures below 30°C. This will result in a loss of the ability to synthesize Asd, which in turn will reduce, and ultimately eliminate, synthesis of DAP. As described earlier, since DAP is an essential constituent of the rigid layer of the bacterial cell wall, the absence of DAP will cause DAP-less death and lysis of cells attempting to grow at temperatures below 30°C.

The *S. typhimurium polA* gene can be isolated from a library of 5 to 7 kb *Sau3A* fragments of *S. typhimurium* DNA cloned into a low copy-number of *polA*-independent vector, such as pSC101. The *polA* clones can be selected for their ability to rescue a *polA*-dependent plasmid when the library is electroporated into a temperature sensitive *polA* mutant χ 1891 and cells are grown at 42°C.

Inverse PCR amplification of the *S. typhimurium polA* gene can then be performed using primers designed to eliminate the promoter region of *polA* but retain the Shine-Dalgarno sequence for translation initiation. The *polA* promoter will then be replaced with the bacteriophage lambda promoter left, which is regulated by the temperature-sensitive *cI857* repressor.

This temperature regulated *polA* cartridge can then be transferred to a *pir*-dependent suicide vector, such as pMEG-011 and electroporated in a *S. typhimurium* host, such as MGN-392. By using *S. typhimurium* strains lacking the *pir* gene required for replication of the suicide vector, the suicide vector, with the temperature regulated *polA* gene, will integrate into the chromosome via a single recombinational event. Subsequent selection against the tetracycline resistance element on the suicide vector using fusaric acid will allow isolation of strains that have undergone a second crossover event replacing the wild-type *polA* gene with the temperature regulated *polA* gene. The resulting strain will thus provide temperature-regulated expression *polA* and temperature-regulated expression of *c2* (from the *cI857PRc2* cartridge

inserted in the defined deletion of *asd*). The ELVS vector pMEG-104, or a derivative with a foreign gene inserted into the multiple cloning site downstream from *P_{trc}*, could then be introduced into the strain. This strain will grow at 37°C. However, at temperatures below 30°C, the strain will undergo DAP-less death due to both the inability to maintain the plasmid vector with the *asd* gene and the inability to express *asd*, and the strain will also lyse due to the expression of phage lysis genes *lys13* and *lys19*.

Example 4: Construction of a chromosomally based Environmentally Limited Viability System.

The same components described in the construction of a vector based thermoregulated Environmentally Limited Viability System can be adapted to make a chromosomally based ELVS. The defined *asd* deletion containing the *cI857PRc2* cartridge can first be introduced into a bacterial strain with properties desirable for its intended use, such as use as a vaccine or in a fermentation to produce a high-value product. The integration of the defined *asd* deletion containing the *cI857PRc2* cartridge can be accomplished using the same procedure as described above for the construction of MGN-392. The thermoregulated *asd lysis* cartridge from pMEG-104 can then be transferred to a suicide vector containing a defined deletion of a selected gene. The chromosomal copy of the selected gene will be the target site of chromosomal integration for the *asd lysis* cartridge. If the host cell is a pathogen, it is preferred that the selected gene be a gene whose inactivation will attenuate the host cell and render it avirulent and immunogenic, as would be the case for a vaccine strain. Such selected genes could be, for example, *cya*, *crp*, *phoP*, *aroA*, *aroC*, *aroD*, *ompR*, *htrA*, and *cdt*. Alternatively, the selected gene could be a dispensable gene so that the engineered strain would have no noticeable defect. For fermentation strains, the selected gene could be chosen such that its inactivation will result in a

fermentation production strain with improved production properties, such as a lessened ability to degrade expressed foreign gene products.

The suicide vector containing the *asd* lysis cartridge inserted into selected gene with the defined deletion can then be electroporated into a strain with a defined *asd* deletion containing the *ci857PRc2* regulatory cartridge. Integration can be accomplished by selecting for first and second recombinational events as described above. The resulting strain will possess all components of the ELVS on the chromosome. If this strain were to be used as a vaccine, the expression of foreign antigens could be achieved by integrating the expression system for that foreign antigen into the chromosome. Alternatively, the gene for the foreign antigen could be contained on a plasmid vector lacking a drug resistance gene but containing a wild-type gene, such as *purA* or *carA*. Such a vector-borne gene would be essential for growth and replication inside an animal host of a vaccine strain with a $\Delta purA$ or $\Delta carA$ chromosomal mutation. Similar considerations could be used to establish a vector system for a fermentation production strain with a chromosomally located ELVS in order to achieve high-level expression of a foreign gene product.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. All patents, patent applications, and publications mentioned herein, whether supra or infra, are hereby incorporated by reference.

We claim:

1. An isolated microbial cell comprising an Environmentally Limited Viability System, wherein the cell is viable in a permissive environment and non-viable in a non-permissive environment, the system comprising

(a) an essential gene, wherein expression of the gene in the cell is essential to the viability of the cell, the essential gene is expressed when the cell is in the permissive environment and is not expressed when the cell is in the non-permissive environment; and

(b) a lethal gene, wherein expression of the gene is lethal to the cell and the lethal gene is expressed when the cell is in the non-permissive environment but not when the cell is in the permissive environment.

2. The cell of claim 1 wherein the permissive environment comprises a temperature of about 37°C and the non-permissive environment comprises a temperature of less than about 30°C.

3. The cell of claim 1 wherein the permissive environment is inside a warm-blooded animal and the non-permissive environment is outside a warm-blooded animal.

4. The cell of claim 1 wherein the essential gene, the lethal gene, or both, is carried on an extrachromosomal vector.

5. The cell of claim 4 wherein the lethal gene is carried on an extrachromosomal vector and expression of the lethal gene is regulated by an expression product of a regulatory gene on a chromosome of the cell.

6. The cell of claim 5 wherein the expression product of the regulatory gene inhibits expression of the lethal gene and is expressed or active only in the permissive environment.

7. The cell of claim 5 wherein the expression product of the regulatory gene induces expression of the lethal gene and is expressed or active only in the non-permissive environment.

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8. The cell of claim 4 wherein the vector has two lethal genes.
9. The cell of claim 8 wherein the vector comprises pMEG-104.
10. The cell of claim 1 wherein the cell is a gram-negative bacterium.
11. The cell of claim 10 wherein the gram-negative bacterium is an enteric bacterium.
12. The cell of claim 11 wherein the genus of the enteric bacterium is selected from the group consisting of *Escherichia* and *Salmonella*.
13. The cell of claim 1 wherein expression of the essential gene is regulated by an expression product of a regulatory gene.
14. The cell of claim 13 wherein the expression product of the regulatory gene inhibits expression of the essential gene and is expressed or active only in the non-permissive environment.
15. The cell of claim 13 wherein the expression product of the regulatory gene induces expression of the essential gene and is expressed or active only in the permissive environment.
16. The cell of claim 4 wherein the system further comprises a replication gene carried on a chromosome of the cell, the expression of which is required for replication of the vector, wherein the replication gene is expressed in the permissive environment and is not expressed in the non-permissive environment.
17. The cell of claim 16 wherein expression of the replication gene is regulated by an expression product of a regulatory gene.
18. The cell of claim 17 wherein the expression product of the regulatory gene inhibits expression of the replication gene and is expressed or active only in the non-permissive environment.
19. The cell of claim 17 wherein the expression product of the regulatory gene induces expression of the replication gene and is expressed or active only in the permissive environment.

20. The cell of claim 1 further comprising an expression gene wherein the expression gene encodes a desired expression product.

21. The cell of claim 20 wherein the desired expression product is an antigen.

22. The cell of claim 21 wherein the antigen is selected from the group consisting of bacterial antigens, viral antigens, plant antigens, fungal antigens, insect antigens, and non-insect animal antigens.

23. The cell of claim 1 for use as a vaccine, wherein the cell is viable when in the an animal and non-viable when outside of the animal, the essential gene is expressed when the cell is in the animal and is not expressed when the cell is outside of the animal, and the lethal gene is expressed when the cell is outside of the animal and is not expressed when the cell is in the animal, wherein the permissive environment comprises a temperature of about 37°C and the non-permissive environment comprises a temperature of less than about 30°C.

24. The cell of claim 23 further comprising an expression gene wherein the expression gene encodes a desired expression product.

25. The cell of claim 24 wherein the desired expression product is an antigen.

26. The cell of claim 25 wherein the antigen is selected from the group consisting of bacterial antigens, viral antigens, plant antigens, fungal antigens, insect antigens, and non-insect animal antigens.

27. A method of making a cell strain with environmentally limited viability comprising stably introducing into a cell

(a) an essential gene, wherein expression of the gene in the cell is essential to the viability of the cell, the essential gene is expressed when the cell is in the permissive environment and is not expressed when the cell is in the non-permissive environment;

(b) a lethal gene, wherein expression of the gene is lethal to the cell and the lethal gene is expressed when the cell is in the non-

32. The method of claim 31 wherein the antigen is selected from the group consisting of bacterial antigens, viral antigens, plant antigens, fungal antigens, insect antigens, and non-insect animal antigens.

33. The method of claim 30 wherein the cell is administered to mucosal surfaces of the animal.

34. The method of claim 33 wherein the mucosal surfaces are in the gastrointestinal tract.

35. The method of claim 30 wherein the essential gene, the lethal gene, or both, is carried on an extrachromosomal vector, and wherein the system further comprises a replication gene carried on a chromosome of the cell, the expression of which is required for replication of the vector, wherein the replication gene is expressed when the cell is in the animal and is not expressed when the cell is outside of the animal.

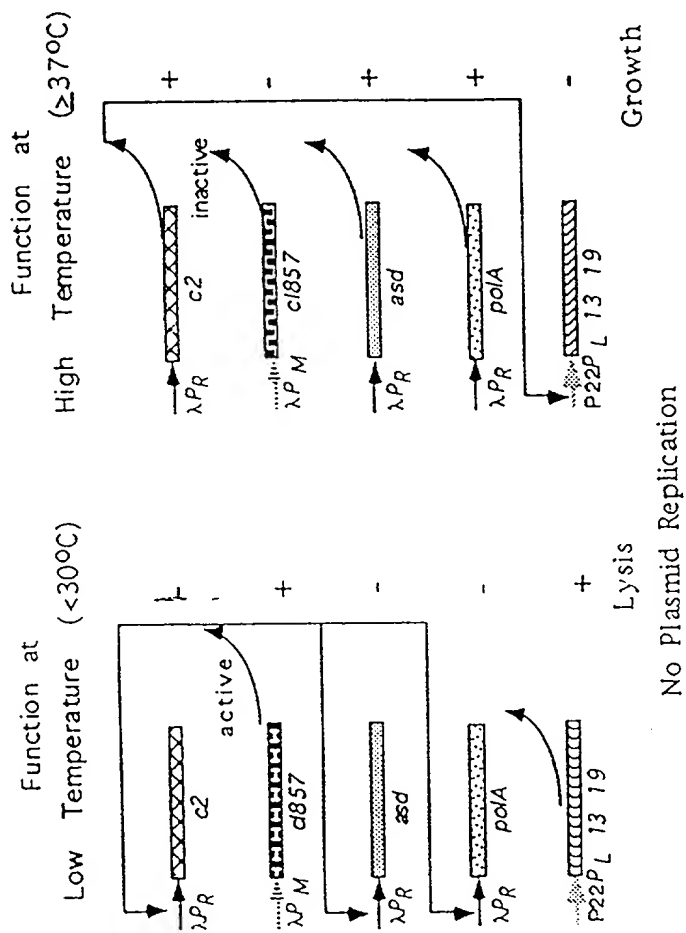
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RECOMBINANT BACTERIAL VACCINE SYSTEM WITH ENVIRONMENTALLY LIMITED VIABILITY

Abstract of the Invention

Disclosed is an Environmentally Limited Viability System (ELVS) for microorganisms based on temperature differences between permissive and non-permissive environments. Viability of the microorganisms are limited to the permissive environment by specifically expressing one or more essential genes only in the permissive environment, or expressing one or more lethal genes only in the non-permissive environment. Environmentally Limited Viability Systems are also disclosed involving coordinate expression of a combination of required genes and lethal genes. Microorganisms containing an Environmentally Limited Viability System are useful for release into a permissive environment. Temperature regulated Environmentally Limited Viability Systems are particularly suited for use with recombinant avirulent *Salmonella* vaccines by limiting their growth to the warmer environment inside the host. Such vaccines can be administered to protect humans or warm-blooded animals against bacterial, viral, mycotic and parasitic pathogens, especially those that colonize on or invade through mucosal surfaces. This antigen delivery system can also be used for expression of gamete-specific antigens to induce immune responses to block fertilization, or to induce immune responses to tumor antigens. In the event that an individual sheds live vaccine into the environment, the presence of the ELVS prevents survival of the vaccine. When environmentally regulated lethal genes are present on an extrachromosomal element and are regulated by chromosomal genes, transfer of the extrachromosomal element to other microorganisms will be limited by unregulated expression of the lethal genes in the recipient microorganism.

Figure 1



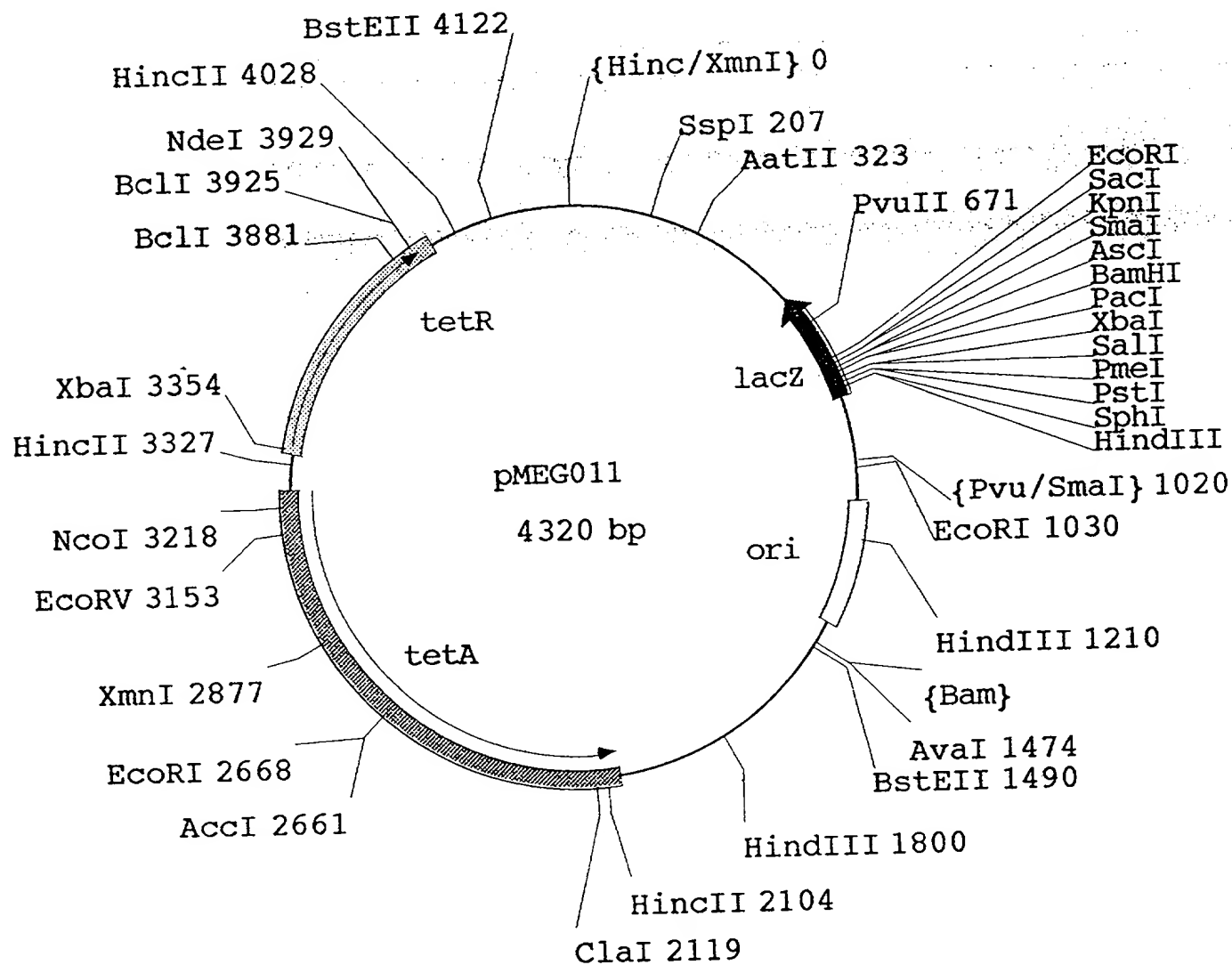


Figure 2
A *pir*-dependent suicide vector

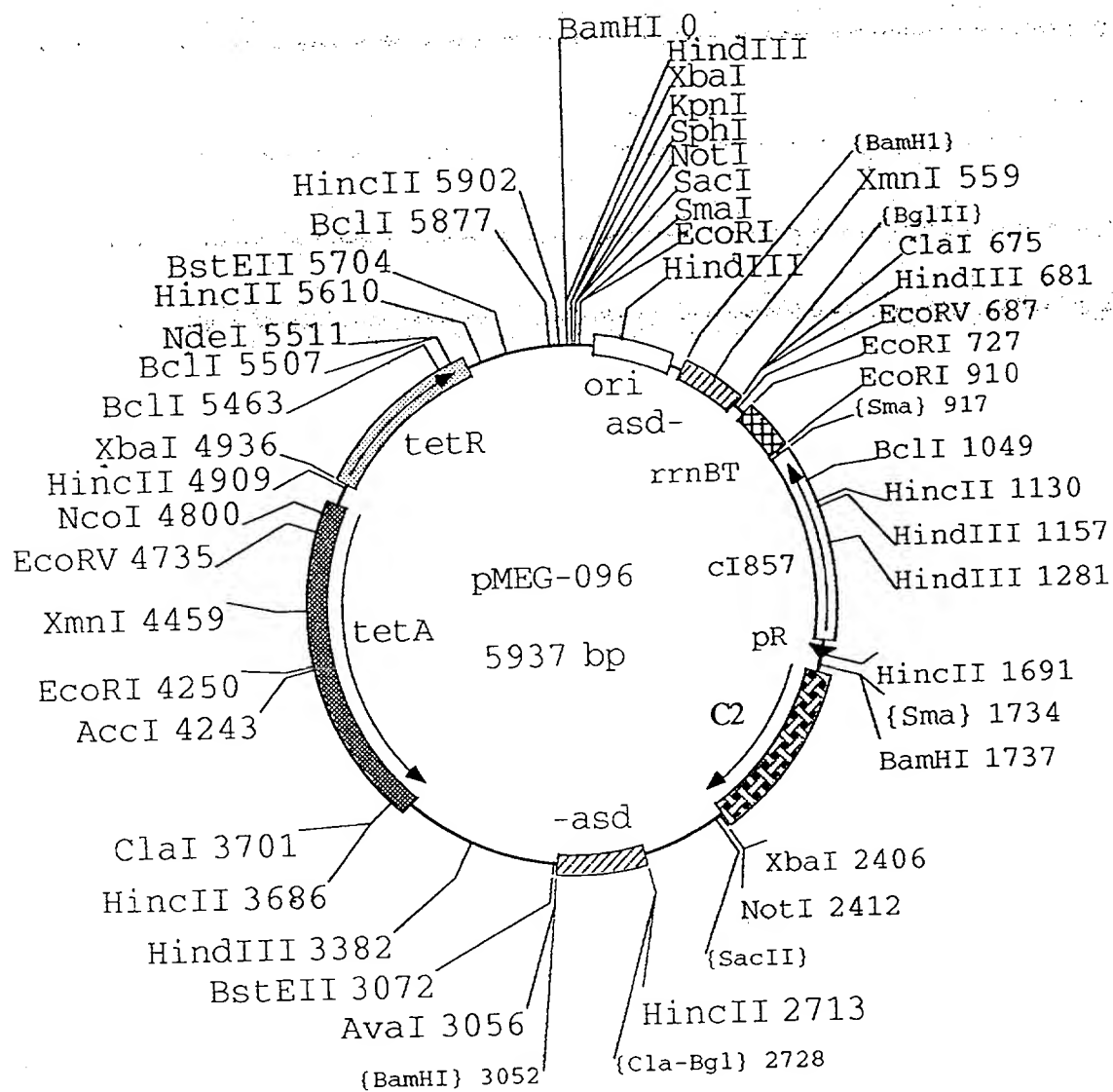


Figure 3

A *pir*-dependent suicide construct for insertion of the *P_R* driven *c2* gene and *cI857* repressor into the *S. typhimurium asd* gene on the chromosome.

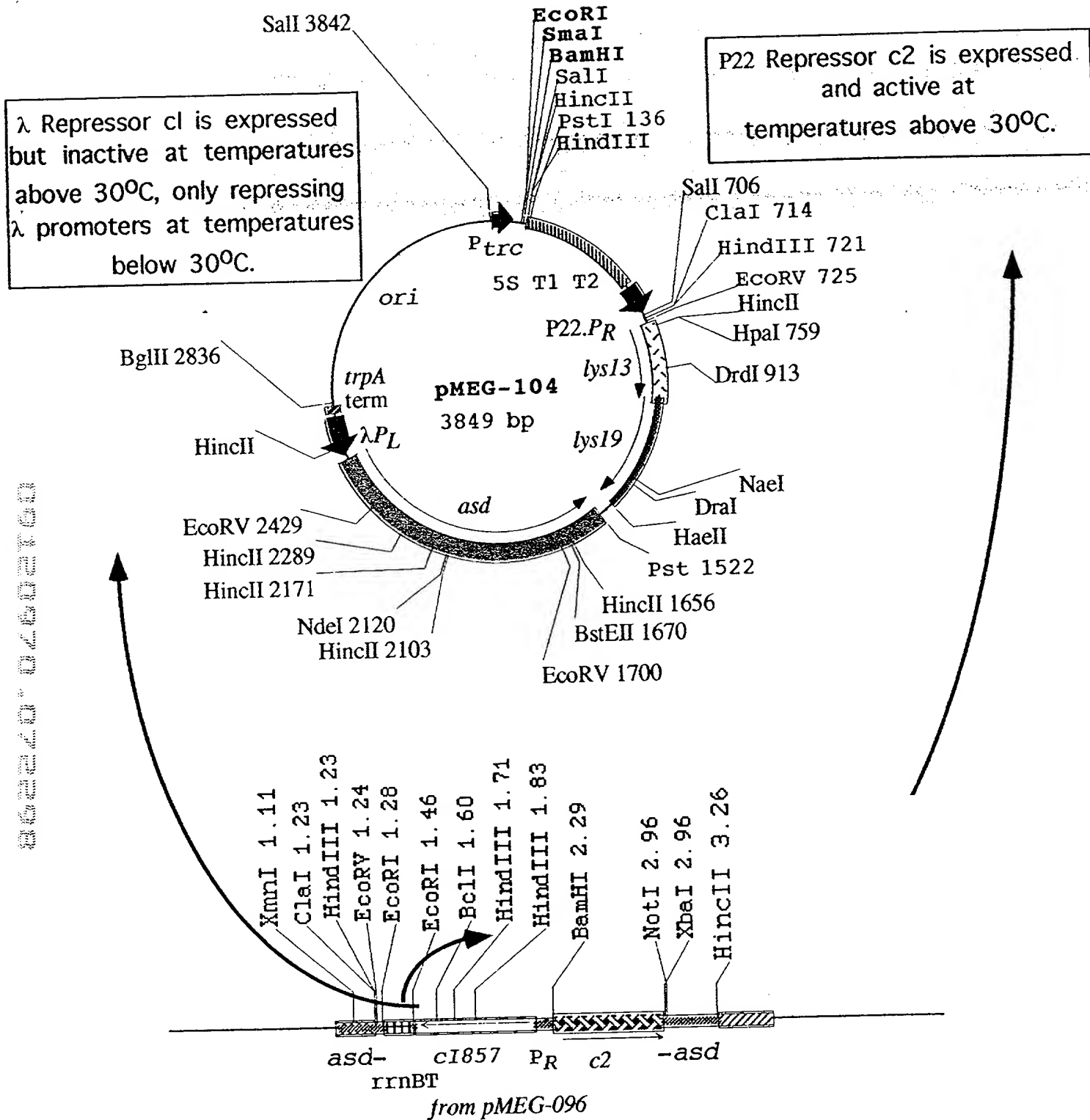


Figure 4
The Environmentally Limited Viability Components in the *S. typhimurium* Strain MGN-417

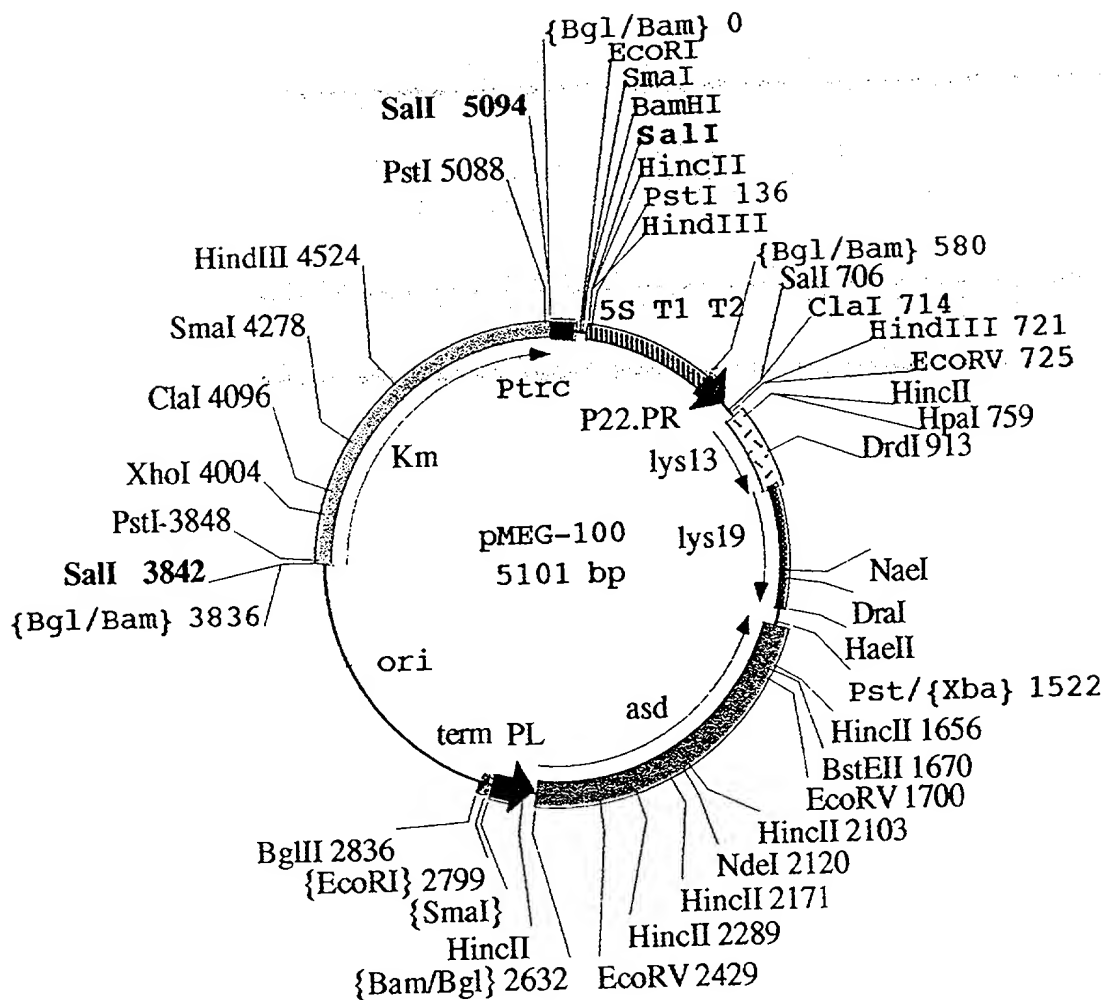
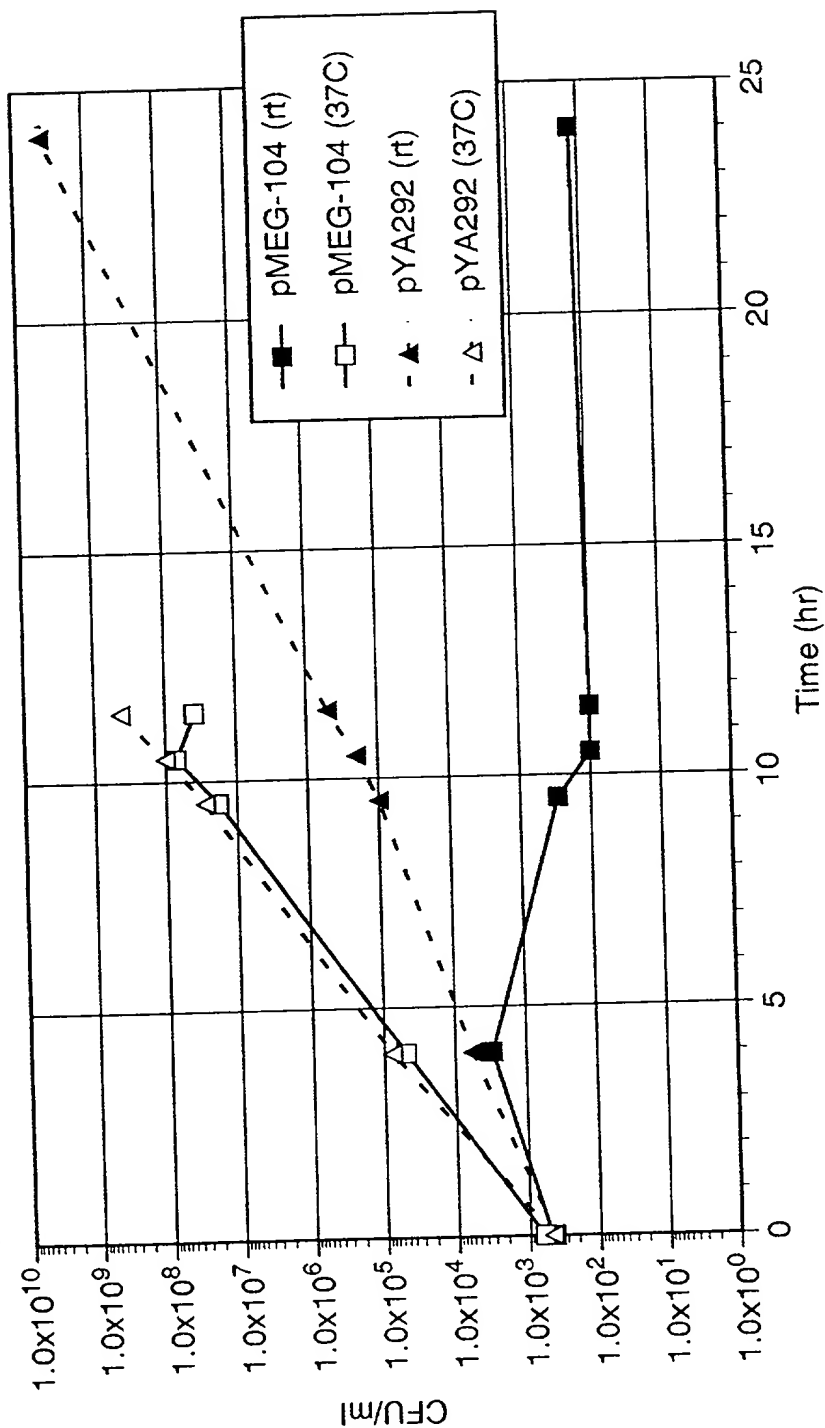


Figure 5

The ELVS expression vector intermediate.

Figure 6
Growth of *S. typhimurium* Environmentally Limited Viability Construct
Relative to Normal *S. typhimurium* with Asd+ Vector



DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below), or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Recombinant Bacterial Vaccine System with Environmentally Limited Viability

the specification of which (check one)

_____ is attached hereto
 _____ was filed on _____
 as application Serial No. _____
 _____ and was amended on: _____
 (if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

| Prior Foreign Application(s) | Priority <u>Claimed</u> |
|--|----------------------------|
| <div style="display: flex; justify-content: space-between;"> <div>_____ (Number)</div> <div>_____ (Country)</div> <div>_____ (Day/Month/Year Filed)</div> </div> | _____ |
| <div style="display: flex; justify-content: space-between;"> <div>_____ (Number)</div> <div>_____ (Country)</div> <div>_____ (Day/Month/Year Filed)</div> </div> | _____ |
| <div style="display: flex; justify-content: space-between;"> <div>_____ (Number)</div> <div>_____ (Country)</div> <div>_____ (Day/Month/Year Filed)</div> </div> | _____ |

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of the application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

| (Application Serial No.) | (Filing Date) | Status (patented, pending, abandoned) |
|--------------------------|---------------|--|
| _____ | _____ | _____ |

"Recombinant Bacterial Vaccine System with Environmentally Limited Viability" Express Mail Label Number TB046533641US
By Roy Curtiss, III and Steven A. Tinge
Filed. June 7, 1995
DECLARATION

(Application Serial No.)

(Filing Date)

Status
(patented, pending, abandoned)

As named inventor, I hereby appoint the following attorney(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

| | |
|----------------------|--------|
| Patrea L. Pabst | 31,284 |
| Madeline I. Johnston | 36,174 |
| Robert A. Millman | 36,217 |

Send Correspondence to: Patrea L. Pabst, Esq.
ARNALL GOLDEN & GREGORY
2800 One Atlantic Center
1201 W. Peachtree Street
Atlanta, GA 30309-3450

Direct telephone calls to: Patrea L. Pabst (404)873-8794

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor Roy Curtiss, III

Inventor's signature Date

Residence 6065 Lindell Blvd, St. Louis, MO. 63112-1009

Citizenship United States

Post Office Address

Full name of second joint inventor (if any) Steven A. Tinge

Inventor's signature Date

Residence 52 Lindenleaf Lane, Belleville, IL 62223

Citizenship United States

Post Office Address

JUL 03 1995

Applicant or Patentee: Roy Curtiss, III, and Steven A.
Tinge Attorney's
Serial or Patent No.: 08/473,789 Docket No.:
MEGAN100

Filed or Issued: June 7, 1995

For: RECOMBINANT BACTERIAL VACCINE SYSTEM
WITH ENVIRONMENTALLY LIMITED VIABILITY

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am

☐ the owner of the small business concern identified
below:

☒ an official of the small business concern empowered to
act on behalf of the concern identified below:

Megan Animal Health, Inc.
3655 Vista Avenue
St. Louis, Missouri 63110

I hereby declare that the above identified small business
concern qualifies as a small business concern as defined in 13
CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of
paying reduced fees under section 41(a) and (b) of Title 35,
United States Code, in that the number of employees of the
concern, including those of its affiliates, does not exceed 500
persons. For purposes of this statement, (1) the number of
employees of the business concern is the average over the
previous fiscal year of the concern of the persons employed on a
full-time, part-time or temporary basis during each of the pay
periods of the fiscal year, and (2) concerns are affiliates of
each other when either, directly or indirectly, one concern
controls or has the power to control the other, or a third party
or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been
conveyed to and remain with the small business concern identified
above with regard to the invention, entitled "Recombinant
Bacterial Vaccine System with Environmentally Limited Viability"
by Roy Curtiss, III, and Steven A. Tinge described in

☐ the specification filed herewith
☒ application serial no. 08/473,789 , filed June 7, 1995
☐ patent no. , issued

"Recombinant Bacterial Vaccine System
with Environmentally Limited Viability"
By: Roy Curtiss, III, and Steven A. Tinge
Filed: June 7, 1995
VERIFIED STATEMENT CLAIMING SMALL ENTITY STATUS

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor concern under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME Washington University
ADDRESS One Brookings Drive, St. Louis, Missouri 63130
☐ INDIVIDUAL ☐ SMALL BUSINESS ☒ NONPROFIT

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: _____

TITLE OF PERSON OTHER THAN OWNER _____

ADDRESS OF PERSON SIGNING _____

SIGNATURE

DATE

Applicant or Patentee: Roy Curtiss, III, and Steven A. Tinge

Attorney's
Docket No.:
MEGAN100

Serial or Patent No.: 08/473,789

Filed or Issued: June 7, 1995

For: RECOMBINANT BACTERIAL VACCINE SYSTEM
WITH ENVIRONMENTALLY LIMITED VIABILITY

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on
behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: Washington University

ADDRESS OF ORGANIZATION: One Brookings Drive
St. Louis, Missouri 63130

TYPE OF ORGANIZATION

- ☒ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
- ☐ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE
(26 USC 501(a) and 501(c)(3))
- ☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE
OF STATE OF THE UNITED STATES OF AMERICA
- ☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE
SERVICE CODE (26 USC 501(a) and 501(c)(3) IF LOCATED
IN THE UNITED STATES OF AMERICA
- ☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL
UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
IF LOCATED IN THE UNITED STATES OF AMERICA

I hereby declare that the nonprofit organization identified
above qualifies as a nonprofit organization as defined in 37 CFR
1.9(e) for purposes of paying reduced fees under section 41(a)
and (b) of Title 35, United States Code with regard to the
invention entitled "Recombinant Bacterial Vaccine System with

09120370.07298

"Recombinant Bacterial Vaccine System
with Environmentally Limited Viability"
By: Roy Curtiss, III, and Steven A. Tinge
Filed: June 7, 1995
VERIFIED STATEMENT CLAIMING SMALL ENTITY STATUS

Environmentally Limited Viability" by Roy Curtiss, III, and
Steven A. Tinge described in

☐ the specification filed herewith

☒ application serial no. 08/473,789 , filed June 7, 1995

☐ patent no. _____ issued _____

I hereby declare that rights under contract or law have been
conveyed to and remain with the nonprofit organization with
regard to the above-identified invention.

If the rights held by the nonprofit organization are not
exclusive, each individual, concern or organization having rights
to the invention is listed below* and no rights to the invention
are held by any person, other than the inventor, who could not
qualify as an independent inventor under 37 CFR 1.9(c) or by any
concern which would not qualify as a small business concern under
37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

***NOTE:** Separate verified statements are required from each named
person, concern or organization having rights to the invention
averring to their status as small entities. (37 CFR 1.27)

NAME: Megan Animal Health, Inc.

ADDRESS: 3655 Vista Avenue, St. Louis, Missouri 63110

☐ INDIVIDUAL ☒ SMALL BUSINESS CONCERN ☐ NONPROFIT ORG.

I acknowledge the duty to file, in this application or
patent, notification of any change in status resulting in loss of

"Recombinant Bacterial Vaccine System
with Environmentally Limited Viability"
By: Roy Curtiss, III, and Steven A. Tinge
Filed: June 7, 1995
VERIFIED STATEMENT CLAIMING SMALL ENTITY STATUS

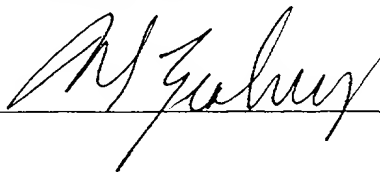
entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: H.S. Leahey
TITLE IN ORGANIZATION: Director, Technology Management
ADDRESS OF PERSON SIGNING: One Brookings Drive, Campus Box 1045
St. Louis, Missouri 63130

SIGNATURE

DATE



8/1/95

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below), or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Recombinant Bacterial Vaccine System with Environmentally Limited Viability

the specification of which (check one)

_____ is attached hereto

 x was filed on June 7, 1995
as application Serial No. 08/473,789

_____ and was amended on: _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

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| Prior Foreign Application(s) | Priority Claimed | | |
|------------------------------|---------------------|------------------------|-------|
| _____ | _____ | _____ | _____ |
| (Number) | (Country) | (Day/Month/Year Filed) | |
| _____ | _____ | _____ | _____ |
| (Number) | (Country) | (Day/Month/Year Filed) | |
| _____ | _____ | _____ | _____ |
| (Number) | (Country) | (Day/Month/Year Filed) | |

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of the application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

| (Application Serial No.) | (Filing Date) | Status (patented, pending, abandoned) |
|--------------------------|---------------|--|
|--------------------------|---------------|--|

"Recombinant Bacterial Vaccine System with Environmentally Limited Viability"
By: Roy Curtiss, III and Steven A. Tinge
Filed: June 7, 1995
DECLARATION

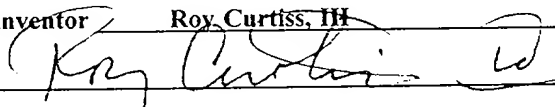
As named inventor, I hereby appoint the following attorney(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

| | |
|----------------------|--------|
| Patrea L. Pabst | 31,284 |
| Madeline I. Johnston | 36,174 |
| Robert A. Millman | 36,217 |

Send Correspondence to: Patrea L. Pabst, Esq.
ARNALL GOLDEN & GREGORY
2800 One Atlantic Center
1201 W. Peachtree Street
Atlanta, GA 30309-3450

Direct telephone calls to: Patrea L. Pabst (404)873-8794

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor Roy Curtiss, III
Inventor's signature  Date 7-6-95
Residence 6065 Lindell Blvd, St. Louis, MO 63112-1009
Citizenship United States
Post Office Address _____

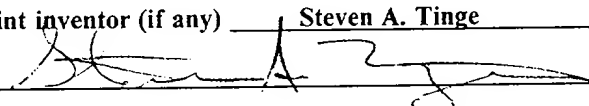
Full name of second joint inventor (if any) Steven A. Tinge
Inventor's signature  Date 7-10-95
Residence 52 Lindenleaf Lane, Belleville, IL 62223
Citizenship United States
Post Office Address _____

Figure 6

Assistant Commissioner for Patents
Washington, D.C. 20231

ASSOCIATE POWER OF ATTORNEY UNDER 37 C.F.R. § 1.34

Sir:

Please recognize as Associate Patent Attorneys and Patent Agent in this case:

| | | |
|------------------|----------|----------|
| Robert A. Hodges | Reg. No. | 41,074 |
| Collen A. Beard | Reg. No. | 38,824 |
| Kevin W. King | Reg. No. | P-42,737 |

Please continue to direct all communication to Patrea Pabst at the following address:

Patrea L. Pabst
ARNALL GOLDEN & GREGORY, LLP
2800 One Atlantic Center
1201 West Peachtree Street
Atlanta, Georgia 30309-3450
(404) 873-8794; 873-8795 (fax)

Date: July 22, 1998

P.L.
Patrea L. Pabst, Reg. No. 31,284

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Atlanta, Georgia 30309-3450
(404) 873-8794
(404) 873-8595 (fax)